Autoantibodies against growth factors and their receptors in fracture healing

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Abstract

The prevalence of autoimmune diseases has constantly increased during the last decades due to demographic, environmental and life style changes. Autoantibodies (aAB) against growth factors and receptors can be causing autoimmune disease but are also found in sera of apparently healthy subjects. Regeneration of bone during fracture healing includes concerted actions of growth factors such as insulin-like growth factor 1 (IGF1) and bone morphogenetic proteins (BMP). Fracture healing is a truly regenerative process reconstituting the bone to its original composition usually within six months. However, some fractures show delayed healing or non-union due to as yet unknown reasons. Neutralizing aAB against growth factors or their receptors might influence and potentially impair the bone healing capacity.

In this study, a cohort of 265 fracture patients with different treatment regimen and healing outcomes, including or not treatment with recombinant human BMP7 (rhBMP7), were analysed. Autoantibodies against IGF1 receptor, insulin receptor, BMP7, BMP2, IGF1 and (pro)insulin were measured in sera of these fracture patients. Three to nine serum samples were available allowing the longitudinal analysis of aAB over a time course of up to one year post surgery. Novel detection assays for the aAB were developed, validated and applied during this work.

The prevalence of aAB against IGF1R and IR was 5% and 6% in fracture patients, respectively. These numbers are in line with the prevalence found in other studies with healthy subjects. The appearance of IGF1R- and IR-aAB was not induced by the surgical intervention and was unrelated to the healing outcome. BMP7-aAB were found in 1-2.5% of healthy subjects and in fracture patients that were not treated with rhBMP7. Patients that had received rhBMP7 treatment showed a higher incidence of BMP7-aAB positivity of 6% at surgery and 18% four weeks post surgery. BMP2-aAB were found in 2% of both healthy controls and BMP7-naïve fracture patients. In the BMP7-treated group, 6% of the fracture patients were identified as BMP2-aAB positive. Only few double positive patients were found. Analysing the BMP7-aAB positivity of five BMP7-aAB positive and seven negative patients over time, aAB were induced after surgery and dropped to undetectable levels in most of the patients indicating a transient induction of BMP7-aAB. Characterizing the biological effect of BMP7-

aAB in a cell-based reporter assay, a neutralizing effect was observed for samples with high titres. Using the same reporter under BMP2 stimulation, the same BMP7-aAB positive samples did not block the signal transduction. Hence, the induced BMP7-aAB are specific for BMP7. Cross-reactivity was detected in one sample only. As the most relevant clinical outcome, the criterion consolidation was analysed defining whether the fracture gap was closed after six months or not. The presence of BMP-aAB was not significantly associated with the healing outcome. IGF1- and insulin-aAB were identified in the fracture patients with a prevalence of 4% and 6%, respectively. Comparable to aAB against the IGF1 and insulin receptors, the IGF1- and insulin-aAB titres were stable in the patients over time. However, in comparison to the BMP7-aAB, the titres were relatively low.

In summary, novel diagnostic assays for the detection and quantification of growth factor and receptor aAB were generated and used to determine aAB in sera from fracture patients. None of the identified aAB were negatively associated with the regeneration process or healing outcome. Ongoing concerns regarding the safety of rhBMP7 treatment are justified as the biological treatment induces aAB against BMP7, and our results indicate that these aAB are biologically active by neutralizing BMP7 signalling. However, since the titres drop within 6 months after induction, the aAB hardly cross-react to the related growth factor BMP2, and no negative effects on the healing outcome were observed, adverse long-term consequences are unlikely. It remains to be analysed, however, if repeated BMP7 treatment leads to stronger and long-lasting immune reactions.

Zusammenfassung

Die Prävalenz von Autoimmunerkrankungen ist in den letzten Jahrzehnten auf Grund von demographischen, Umwelt- und Lebensstilveränderungen stark angestiegen. Autoantikörper (aAB) gegen Wachstumsfaktoren und deren Rezeptoren können in Autoimmunerkrankungen krankheitsverursachend sein; sie wurden aber auch in vermeintlich gesunden Menschen detektiert. Die Regeneration von Knochen während der Frakturheilung beinhaltet das Zusammenspiel von Wachstumsfaktoren, wie z.B. Insulin-like Growth Factor 1 (IGF1) und Bone Morphogenetic Proteins (BMP). Im Normalfall schließt sich der Frakturspalt innerhalb von sechs Monaten, wobei es allerdings in einigen Patienten zu einer verzögerten oder unvollständigen Heilung kommen kann. Die Gründe hierfür sind bisher nicht komplett verstanden. Neutralisierende aAB gegen Wachstumsfaktoren oder deren Rezeptoren könnten den Heilungsprozess verzögern und potentiell beeinträchtigen

In dieser Arbeit wurden 265 Frakturpatienten analysiert, die unterschiedliche Heilungsergebnisse hatten. Einige der Patienten hatten eine Behandlung mit rekombinantem humanem BMP7 (rhBMP7) erfahren. Drei bis neun Serumproben waren pro Patient verfügbar, was eine longitudinale Analyse über einen Zeitverlauf von bis zu einem Jahr nach der Operation ermöglichte. Autoantikörper gegen IGF1 Rezeptor, Insulin Rezeptor, BMP7, BMP2, IGF1 und (Pro)Insulin wurden in den Seren dieser Frakturpatienten gemessen. Hierfür wurden im Rahmen dieser Arbeit neuartige Detektions-Assays entwickelt, validiert und angewendet.

In Frakturpatienten wurden in 5% der Seren aAB gegen den IGF1R und in 6% gegen den IR gefunden. Diese Prävalenzen stimmen mit den Ergebnissen in vorherigen Studien mit gesunden Probanden überein. Das Auftreten von IGF1R- und IR-aAB wurde nicht durch die chirurgische oder pharmakologische Intervention induziert und war nicht mit dem Heilungsergebnis assoziiert. BMP7-aAB wurden in 1-2,5% gesunder Probanden und Frakturpatienten, die nicht mit rhBMP7 behandelt wurden, detektiert. Patienten, die mit rhBMP7 behandelt wurden, zeigten ein höheres Auftreten der BMP7-aAB Positivität mit 6% zum Zeitpunkt der Operation und 18% vier Wochen nach der Operation. BMP2-aAB wurden in 2% der gesunden Kontrollen und in BMP7-naiven Patienten bestimmt. In der BMP7-behandelten Gruppe wurden 6% als BMP2-

aAB positiv identifiziert. Nur wenig doppelt positive Patienten wurden gefunden. Bei der Untersuchung der BMP7-aAB Positivität von fünf BMP7-aAB positiven und sieben negativen Patienten über den Zeitverlauf zeigte sich, dass die aAB nach der Operation induziert werden und die Titer in den meisten Patienten wieder auf ein nicht-detektierbares Level abfallen, was auf eine transiente Induktion der BMP7-aAB hin weist. Bei der Charakterisierung des biologischen Effekts der BMP7-aAB durch einen zell-basierten Reporter-Assay, zeigte sich ein neutralisierender Effekt in Proben mit hohem BMP7-aAB Titer. Bei der Anwendung des gleichen Reporters unter BMP2-Stimulierung zeigten dieselben BMP7-aAB positiven Proben keine Blockierung der Signaltransduktion. Die BMP7-aAB sind daher spezifisch gegen BMP7 gerichtet. Kreuzreaktivität wurde nur in einer Probe detektiert. Als das wichtigste Kriterium für klinische Relevanz wurde die Konsolidierung untersucht, welche angibt, ob der Frakturspalt nach sechs Monaten geschlossen ist oder nicht. Das Vorhandensein von BMP-aAB wurde nicht signifikant mit der Konsolidierung in Zusammenhang gebracht. IGF1- und Insulin-aAB wurden in 4% bzw. 6% der Frakturpatienten gefunden. Vergleichbar zu den aAB gegen IGF1- und Insulin-Rezeptor, waren die IGF1- und Insulin- aAB Titer stabil über die Zeit. Allerdings waren die Titer im Vergleich zu den BMP7-aAB relativ niedrig.

Zusammenfassend wurden neue diagnostische Assays zur Detektion und Quantifizierung von aAB gegen Wachstumsfaktoren und deren Rezeptoren generiert und angewandt um aAB in Seren von Frakturpatienten zu messen. Keiner der identifizierten aAB war negativ mit dem Heilungsprozess assoziiert. Bedenken bezüglich der Sicherheit von rhBMP7 Behandlungen sind berechtigt, da die Anwendung aAB gegen BMP7 induziert und unsere Ergebnisse darauf hinweisen, dass diese aAB biologisch aktiv sind, indem sie den BMP7-Signalweg blockieren. Da die Titer innerhalb von sechs Monaten nach Induktion wieder abfallen, die aAB kaum mit dem verwandten Protein BMP2 kreuzreagieren und kein negativer Effekt auf das Heilungsergebnis gefunden wurde, sind negative Langzeitfolgen unwahrscheinlich. Es bleibt jedoch zu untersuchen, welchen Einfluss eine wiederholte Gabe von rhBMP7 hat und ob diese zu einer stärkeren und anhaltenden Immunreaktion führt.

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Abbreviations

°C	degree Celsius
µ	micro
µm	micro meter
A	ampere
aAB	autoantibodies
AB	antibody
ADA	anti-drug antibodies
AIRE	Autoimmune Regulator
ALK	activin-like receptor kinase
AMA	anti-mitochondrial antibodies
APC	antigen-presenting cell
approx.	approximately
APS	ammonium persulfate
ATP	adenosine triphosphate
BISC	BMP-induced signalling complex
BMP	bone morphogenetic proteins
BMU	basic multicellular unit
bp	base pairs
BRE	BMP-responsive-element
BSA	bovine serum albumin
ca.	circa
CIA	chemiluminescence immunoassays
CD	cluster of differentiation
Cre	enzyme causing recombination
cv	coefficient of variation
d	deci
Da	Dalton
DMEM	Dulbecco`s MEM
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
E.coli	*Escherichia coli*
e.g.	for example
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assays
EPO	erythropoietin
et al.	et altera
FBS	foetal bovine serum
FGF23	Fibroblast Growth Factor 23
g	gram
G418	geneticin
GDF	growth-and-differentiation factor
HEK	human embryonic kidney
HLA	human leukocyte antigen
HRP	horse-raddish peroxidase

HSC	hematopoietic stem cell
i.e.	id est (that is to say)
ICA	islet-cell antibodies
IFN	Interferon
Ig	immunoglobulin
IGF1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor 1 receptor
IGFBPs	IGF binding proteins
IQR	interquartile range
IR	insulin receptor
k	kilo
L	litre
LID	liver-specific *igf1* gene-deletion mouse model
LUC	firefly luciferase
M	molar
MACN	acridiniumester-N-hydroxy-succinimid
MAPK	mitogen-activated protein kinase
MCS	multiple cloning site
M-CSF	macrophage colony-stimulating factor
MHC	major histocompatibility complex
min	minutes
ml	milli litre
MSC	mesenchymal stem cell
n	nano
n.d.	not determined
neg	negative
o.n.	overnight
op	operational procedure
Opg	osteoprotegerin
Osx	osterix
p	*p*-value
$P_{0.75}$	75th percentile
PAMPs	pathogen-associated molecular patterns
PBC	primary biliary cirrhosis
PBS	phosphate-buffered saline
PEI	polyethylenimine
PFC	pre-formed complex
pos	positive
PRRs	pattern-recognition receptors
R	receptor
RA	rheumatoid arthritis
RANK	Receptor Activator of NF-κB
RANK-L	RANK ligand
rel	relative
RF	rheumatoid factor
rh	recombinant human
RIA	reamer irrigator aspirator

RLU	relative light units
rpm	rotation per minute
RT	room temperature
Runx2	runt-related transcription factor 2
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEAP	secreted alkaline phosphatase
SLE	systemic lupus erythematosus
SPR	surface plasmon resonance
T75	75 cm^2 flasks
TAE	TRIS-Acetate-EDTA
TBST	Tris-buffered saline with Tween20
TEMED	tetramethylethylenediamine
TGF	transforming growth factor
THPO	thrombopoietin
TRAK	TSH-receptor autoantibodies
TSH	thyroid-stimulating hormone
U	unit
UV	ultra violet
V	volt
w	weeks
w/o	without

1 Introduction

1.1 Autoimmunity

1.1.1 Function of antibodies and generation of diversity

The immune system is of central importance in order to protect an organism from pathogens and to actively fight a pathogenic infection. The innate immunity provides a set of inherited tools to act as a first line defence mechanism, whereas the adaptive immunity (alongside providing memory for later encounters with the same pathogen) provides for a more specific and induced immune response. As part of the adaptive immunity, antibodies play an important role in the defence against pathogens and can react against their antigen in a very specific manner. Classical effector mechanisms of antibodies as humoral immunity opposed to cellular immunity are: neutralization of toxins, opsonisation of bacteria to make them detectable by phagocytes and activation of the complement cascade which in the end lyses the bacterium (Figure 1-1). How such a diversity in antibody specificity can be realized occupied immunologists for a long time.

Antibodies are produced by B cells as B cell receptors. Essential in the understanding of B cell activation was the discovery that one B cell produces only one type of antibody with one unique antigen specificity (Nossal and Lederberg, 1958). Sir Frank Macfarlane Burnet with his clonal selection theory contributed immensely to understanding the generation of a huge diversity in antibody specificity (Burnet, 1959). In summary, it states that one lymphocyte carries only one specific receptor. The corresponding antigen activates the B cell from a pre-existing pool by interaction with its receptor leading to receptor signal transduction. The B cell is clonally expanded and produces antibodies of the same specificity as the receptor. The huge diversity in B cell receptor repertoire is achieved during their development and maturation by V, D and J gene recombination for the heavy chain, V and J gene recombination for the light chain and the combination of heavy and light chain (Tonegawa, 1983).

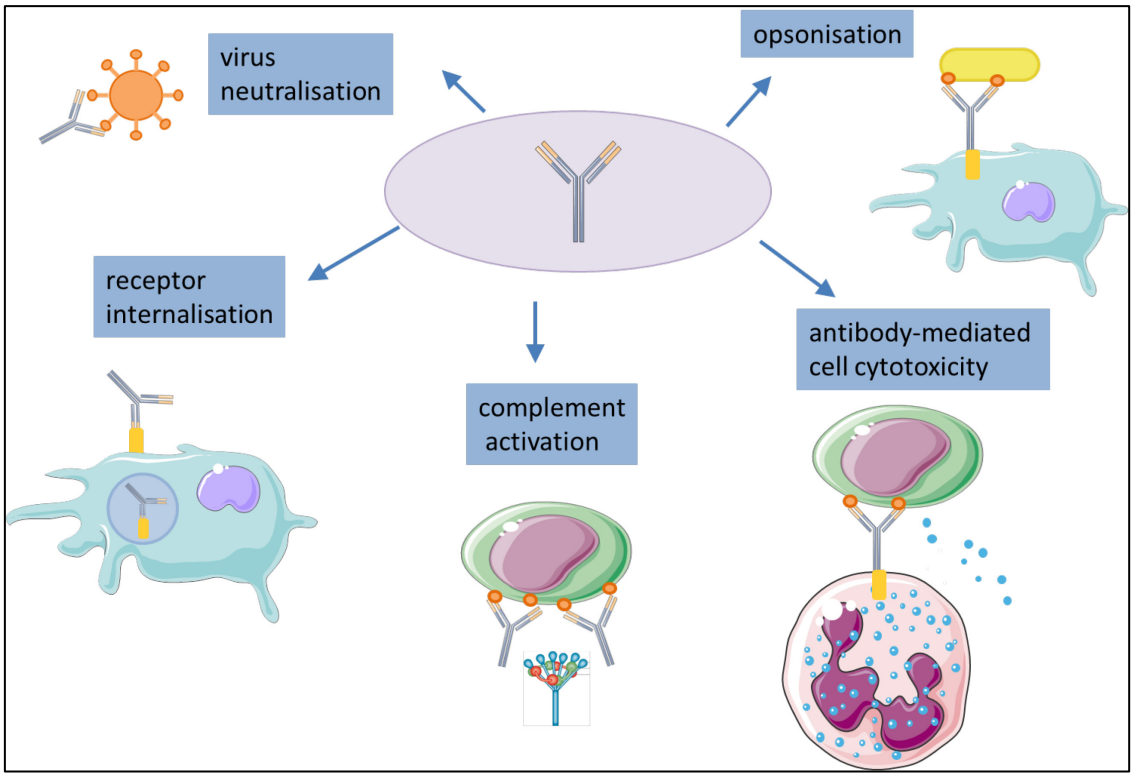

Figure 1-1: Function of antibodies. Modified after (Spasevska, 2014) and produced using Servier Medical Art.

1.1.2 The establishment of self-tolerance

If receptor diversity is provided by a random process of genetic recombination, how is self-reactivity prevented? The mechanistic understanding of tolerance goes hand in hand with understanding how B cells are activated. Paul Ehrlich´s statement of “Horror Autotoxicus” suggested that the immune system can only be directed against foreign substances which rendered the occurrence of autoimmunity impossible *per se*. It took over 50 years to be revised and replaced by Burnets clonal selection theory where he also stated that self-reactive lymphocytes are deleted at an early stage. This proposal based on the finding that non-identical cattle twins carried blood cells of their twin and were tolerant of them (Owen, 1945). Experimentally, Peter Brian Medawar showed that when mice were injected with donor cells as babies they would tolerate skin grafts from those donors when they are adult (Billingham et al., 1953). Burnet and Medawar were given the Nobel Prize in 1960 for their discovery of acquired immunological tolerance. Since then the Self-Nonself discrimination model was accepted and became predominant in the immunologists’ opinion. The discovery of change in antibody specificity by somatic hypermutation required

further explanation why self-reaction does not occur more often. Bretscher and Cohn proposed a second signal for the induction of B cells besides antigen-receptor interaction. They suggested that help signals are required from a helper cell which is another antigen-specific cell and we know today is a helper T cell. Without this second signal the B cell would undergo apoptosis if it received a positive signal from its B cell receptor (Bretscher and Cohn, 1970). As a reaction to the discovery that T cells respond more strongly to foreign cells of their own species than to cells of other species, Lafferty and Cunningham added another pixel to the picture. In order to be activated, they proposed that T cells need another (species-specific) co-stimulatory signal which they receive from stimulatory cells (Lafferty and Cunningham, 1975). Today we call these cells antigen-presenting cells (APCs). How these cells help to discriminate between foreign and self in the induction of antibody production became clearer when germ-line encoded pattern-recognition receptors (PRRs) were discovered to be present on APCs. These PRRs bind evolutionary conserved structures on pathogens, so-called pathogen-associated molecular patterns (PAMPs) (Janeway et al., 1989). When APCs are activated via their PRRs they signal costimulatory signals to T cells, process pathogenic antigens and present them to T cells. These do not only allow discrimination between self and nonself but between “infectious-nonself” and “non-infectious-self” (Janeway, 1992). Furthermore, PRRs on APCs “provide broad spectrum recognition of harmful foreign materials” (Janeway, 1992). The broad detection of pathogens then leads to specific activation of antibody production against pathogenic antigen. Without the signals from APCs, T cells are not activated. Without the signals from T cells, B cells are not activated but tolerated (summarized in Figure 1-2). This model still leaves open questions why transplants are rejected or why autoimmunity occurs. New concepts of immunogenicity have been proposed. The Danger Model by Polly Matzinger suggests that activation occurs upon danger signals from injured cells that can be infected cells or cells in necrosis or otherwise unhealthy cells (Matzinger, 2002). This model elegantly explains why a foetus is foreign but tolerated and a transplant is rejected. Matzinger extents her theory in that the immune system does not only need to know when to respond but how (which effector) to react. She sees the answer in the tissue itself that sends healthy signals to be tolerated or danger signals to induce

immune reaction and also determines the proper immune reaction. Another proposition is the criterion of continuity (Pradeu and Carosella, 2006) which states that the immune system reacts upon a sudden increase in antigen availability and tolerates continuous antigen concentrations.

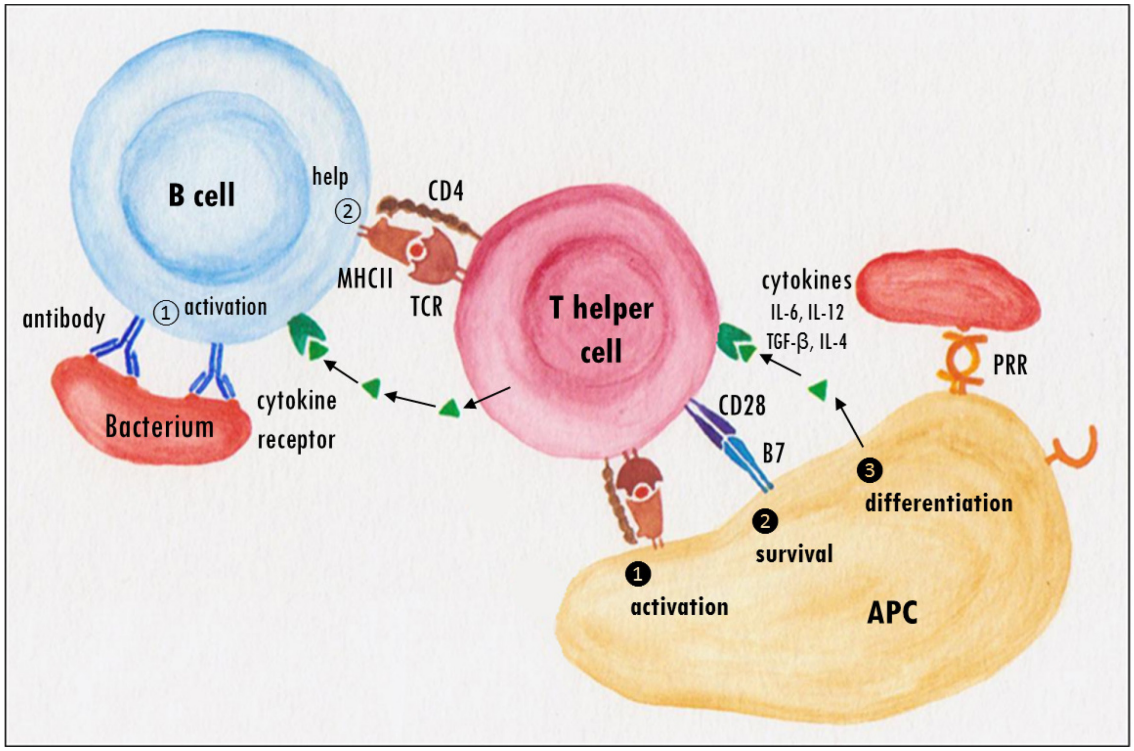

Figure 1-2: B cell activation. Antigen-presenting cells process antigens and present them in MHC complexes on their surface. The APC provides three signals for the developing T cell: activation, survival and differentiation. The B cell requires two signals to be activated; activation by B cell receptor interaction with the antigen and help signal provided by the T helper cell. APC: antigen-presenting cell, TCR: T cell receptor, PRR: pattern-recognition receptor, MHC: major histocompatibility complex. Figure produced by Gesa Nöhren, modified after (Murphy, 2012).

The challenge for the immune system is to provide defence mechanisms against potentially all foreign invaders, whilst at the same time tolerate the organism itself as well as all foreign but harmless substances. In order to achieve self-tolerance several control mechanisms exist during the development of B and T lymphocytes. In general, we discriminate between central and peripheral tolerance. Central tolerance is carried out in the central lymphoid organs (bone marrow for B cells and thymus for T cells) where lymphocytes develop. If B cells show no or low autoreactivity, they are allowed to maturate. B cells with autoreactivity can have different fates (Figure 1-3). Strong autoreactive B cells are eliminated by apoptosis (clonal deletion). B cells

with low autoreactivity can undergo receptor editing by light chain rearrangement. Another possibility is anergy, a permanent state of unresponsiveness which in the end leads to death because these B cells will never be stimulated by T cells. The fourth option is immunological ignorance. This occurs when the antigen is not accessible in the bone marrow or weak binding of the antigen does not generate an activating signal. These cells, however, can be activated if conditions change.

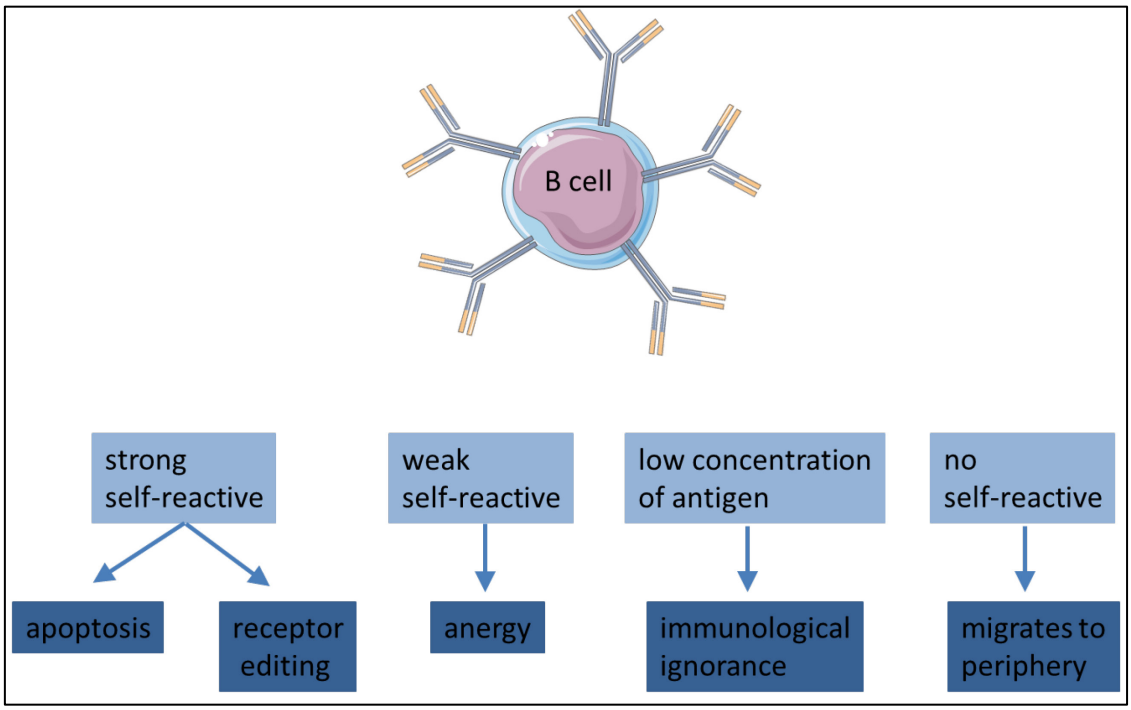

Figure 1-3: B cell fates during development. B cells with strong self-reactive receptors either die by apoptosis or their receptors are changed by receptor editing. Weak self-reactive B cells go into anergy, a state of permanent unresponsiveness. B cells that receive low signalling due to low concentrations of antigen survive in a state of immunological ignorance. B cells with no self-reaction are allowed to mature. Figure was produced using Servier Medical Art.

Peripheral tolerance is induced when lymphocytes start to circulate. The default fate for B cells is death; therefore they survive only approximately three days when they are not positively selected. Positive selection occurs by inflammation, i.e. pathogen-derived antigens, co-stimulatory molecules and cytokines. Without inflammation, the lymphocytes that encounter their antigen are inactivated. A pathogenic infection is a sudden increase in antigen concentration. Therefore, naive lymphocytes are activated upon a sudden increase in antigen-receptor signal, whereas they are tolerated in the presence of constant antigen concentrations (Murphy, 2012).

All in all, the self-tolerance mechanisms are neither strict nor perfect. All pathways are indirect; none is able to autonomously discriminate between self and foreign. This favours on the one hand the maintenance of a large and diverse repertoire of B cell receptors but on the other hand allows low affinity autoreactivity to survive.

1.1.3 Triggers of autoimmunity

When tolerance mechanisms somehow are overcome, autoimmunity develops. But what is it that determines why autoimmunity develops in some individuals and not in others? Without doubt autoimmunity has a multifactorial origin. This includes a genetic, environmental and hormonal component, immune defects and other aspects. Yehuda Shoenfeld elegantly termed this interplay as the "mosaic of autoimmunity" (Shoenfeld and Isenberg, 1989) (Figure 1-4). A mosaic consists of the arrangement of several different pieces in order to create a full picture. Arranging the pieces in a different order produces a different picture. Shoenfeld uses this as an analogy for autoimmunity as different endogenous and external factors collectively interact and affect autoimmune disease risk and course. The underlying molecular reasons for developing the same autoimmune disease may differ profoundly between individuals as well as different subjects with the same disease may show differing phenotypes and progressions.

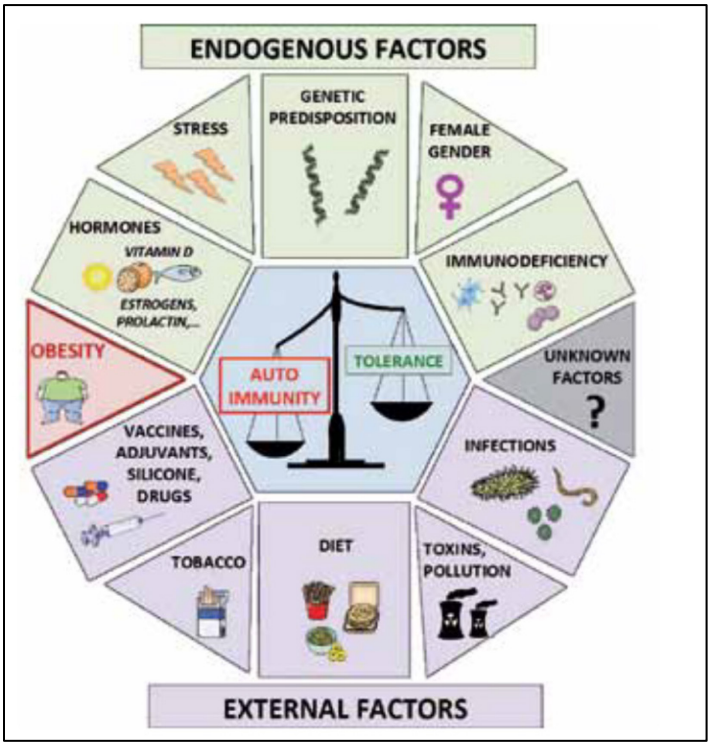

Figure 1-4: The mosaic of autoimmunity. A multitude of factors contribute to the risk and cause of autoimmunity with different combinations of the aspects in each individual leading to a different clinical picture. The factors involved can be divided into endogenous factors, like genetic predisposition (e.g. HLA type), gender, hormone levels and external factors, such as life style (smoking, diet, etc.) and/or environmental triggers (infections, toxins, etc.) (Versini et al., 2014).

An important genetic component of predisposition to develop autoimmunity is the individual genotype of the human leukocyte antigen (HLA) class II molecules (Gregersen, 1990). Additionally, many other polymorphisms in immune-system or organ-specific genes have been identified to play a predisposing role. The gene *Autoimmune Regulator* (*AIRE*) plays an important role in negative selection during the development of T cells by driving the expression of a large number of tissue-specific antigens in the primary lymphoid tissues. It is therefore not surprising, that *AIRE* -/- mice are prone to develop autoimmune disease (Campbell et al., 2009).

As opposed to the genetic component, infections can be an environmental trigger of autoimmunity. Viral, bacterial and parasitic infections have been associated with triggering autoimmune diseases. Epstein-Barr Virus infection was correlated to the development of Systemic lupus erythematosus (SLE) (James et al., 2001). Infections can induce autoimmunity by different mechanisms; molecular mimicry, epitope spreading, bystander activation or

viral persistence. Bacterial infection can precede autoimmune disease. About 50% of reactive arthritis cases are associated with prior *Chlamydia trachomatis* infection (Keat et al., 1987). Other bacterial infections have also been linked to reactive arthritis and bacterial antigens were found to be present in the synovial fluid of reactive arthritis patients. Infections in autoimmunity have somehow a double-role as they were both associated positive and negative with the incidence of autoimmune diseases. The dramatic increase in autoimmune diseases and allergies during the last decades was linked to the epidemiological trend of decreased childhood infections in developed countries due to improved hygiene (Bach, 2002) (Figure 1-5). This so-called “hygiene hypothesis” was first suggested in 1968 (Greenwood, 1968) and proved of relevance for allergy and hygiene in relation to number and order of siblings (Strachan, 1989). Infections, especially during childhood, appear to prevent from allergy and autoimmune disease.

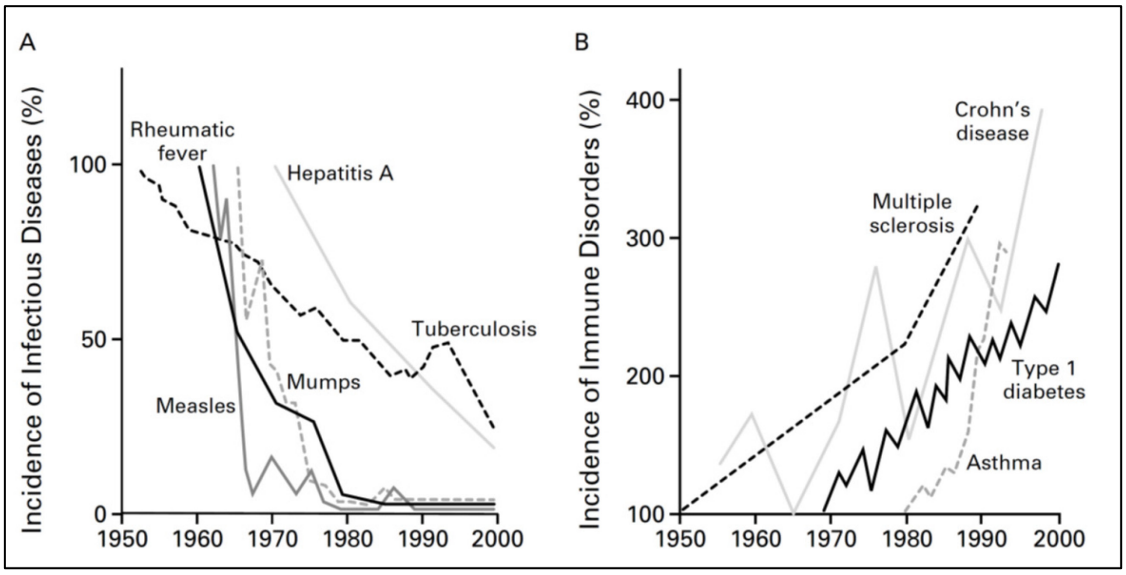

Figure 1-5: The hygiene hypothesis. (A) The incidence of infectious diseases, especially in childhood, has rapidly decreased in the 50 years between 1950 and 2000. (B) In the same period of time allergies and autoimmune diseases increased resulting in the hypothesis that the encounter with infectious agents protects from dysregulation of the immune system (Bach, 2002).

Even today allergies and autoimmune diseases are practically absent in undeveloped countries with poor hygiene and high personal exposure rates to infectious agents. The search for the germs that we were exposed to before, but were subsequently removed by the introduction of hygienic means, pointed

towards parasitic worms. Helminths co-evolved together with their hosts and are able to modify the host immune system in a way that prevents it from becoming easily activated without suppressing its activity too much as to let the host die from other infections. Therefore, helminths seemed to play an important positive immune regulatory role. The therapeutic application of helminths was successfully conducted in human clinical trials for inflammatory bowel disease, multiple sclerosis and celiac disease and also in animal trials for several additional diseases, reviewed in (Versini et al., 2015).

More and more pieces have been added to the mosaic of autoimmunity, like obesity (Versini et al., 2014), smoking (Perricone et al., 2016), female gender (Gleicher and Barad, 2007), vaccines and adjuvants (Guimaraes et al., 2015). However, the emerging picture is still far from being complete.

1.1.4 Autoantibodies in health and disease

As the tolerance mechanisms are not perfect, auto-reactive lymphocytes mature under some circumstances to antibody-producing plasma cells or auto-reactive T cells. Autoantibodies (aAB) are characteristic for many autoimmune diseases. A well investigated example of an autoimmune disease with aAB against receptors is Graves´ disease, also known as Morbus Basedow, since it was described independently by Robert James Graves (Graves, 1835) and Carl von Basedow (Von Basedow, 1932). Thyroid stimulating hormone (TSH) receptor autoantibodies (TRAK) cause receptor activation and stimulate the thyroid gland to produce thyroid hormone (Figure 1-6 A, B). Negative feedback of thyroid hormones to reduce TSH production by the pituitary usually causes downregulation of thyroid hormone biosynthesis and release. In Graves´ disease, the autoantibodies constantly stimulate the TSH receptor, thereby interrupting this feedback loop leading to hyperthyroidism.

Another example of aAB against receptors, but this time with an inhibiting effect, is the autoimmune disease Myasthenia gravis (C, D). Autoantibodies against the acetylcholine receptor lead to internalisation and digestion of the receptors. Eventually, nerve impulses are no longer propagated and muscle contraction cannot occur (Fambrough et al., 1973; Drachman 1994).

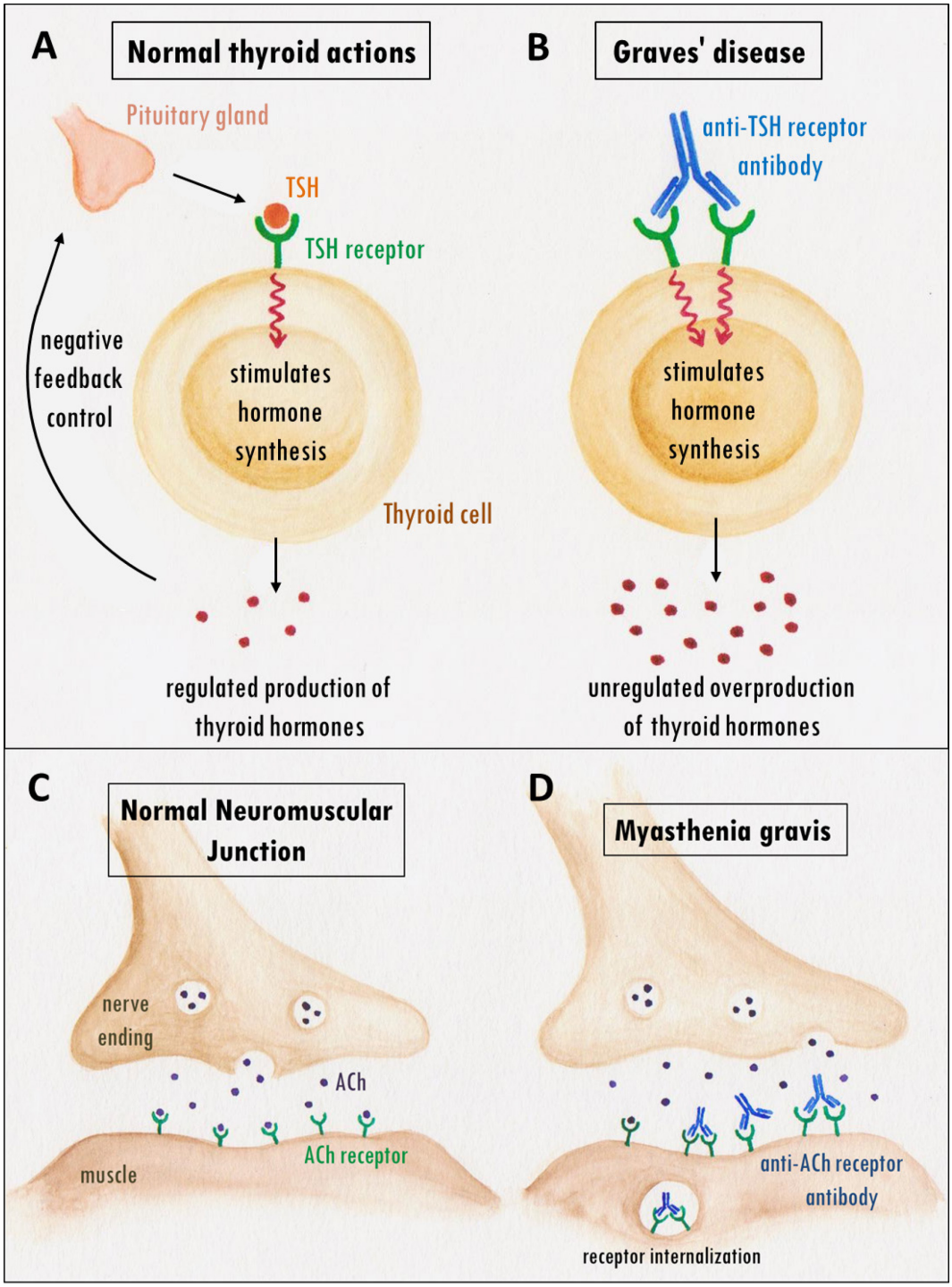

Figure 1-6: Pathology of autoimmune diseases. (A) Under healthy conditions the pituitary gland secretes TSH which stimulates the thyroid via TSH receptor activation to produce thyroid hormones which in turn provide a negative feedback loop acting on the pituitary to stop secreting TSH. (B) In case of Graves' disease stimulating autoantibodies against the TSH receptor constantly induce the secretion of thyroid hormones without stimulation by TSH. (C) At the neuromuscular junction activation of acetylcholine receptors by acetylcholine leads to muscle contraction. (D) In Myasthenia gravis autoantibodies against the acetylcholine receptor lead to internalization and degradation of the receptors with the result that neuronal signal cannot be propagated and muscle contraction does not occur. TSH: thyroid stimulating hormone, ACh: acetylcholine. Figure produced by Gesa Nöhren and modified after (Murphy, 2012).

Whether aAB alone are sufficient to cause a disease is tested by passive transfer into animal models that then may show the characteristic disease symptoms. Transfer of IgG fraction from Myasthenia gravis patients´ sera into mice led to similar symptoms, e.g. reduced acetylcholine receptors at neuromuscular junctions (Toyka et al., 1975).

Autoantibodies against the Insulin-like growth factor 1 receptor (IGF1R) have been identified in 10% of Graves' disease patients and healthy controls indicating a presence of aAB in the normal population without obvious disease symptoms (Minich et al., 2012).

Detection of aAB is often possible long before the clinical onset of an autoimmune disease. In U.S. Armed Forces personnel 115 out of 130 SLE-patients had SLE-aAB before disease diagnosis (Arbuckle et al., 2003). Similarly, for primary biliary cirrhosis (PBC) anti-mitochondrial antibodies were found in individuals with normal liver function, they predict PBC and serve as early diagnostic markers (Metcalf et al., 1996). Along this line, rheumatoid factor (RF) can be detected in subjects without rheumatoid arthritis (RA) and RF-titre was found to be a risk factor for progression to RA in a longitudinal epidemiologic study (Puente et al., 1988). The risk of developing type 1 diabetes correlates to islet-cell antibodies (ICA), especially the combination of different ICA against several antigens (Verge et al., 1996).

In summary, autoantibodies can be the disease-causing agents, bystanders in autoimmune disease and are often found in apparently healthy individuals without clinical importance or as early predictive biomarker for the onset of the autoimmune disease.

1.1.5 Anti-drug antibodies against biologicals

Drugs have the potential to induce unwanted immune reactions thereby hindering the pharmacological efficacy of said drugs. The formation of anti-drug antibodies (ADA) is of concern because it can interfere with the safety and the efficacy of the drug. ADA can bind the drug, form immune complexes and thereby enhancing the clearance of the drug, thus requiring higher dosages for effective treatment. Neutralizing ADA directly inhibit the pharmacological activity of the drug usually by preventing it from binding to target structures. The clinical use of biologicals as treatment options has proven highly successful and

generally being less immunogenic than synthetic compounds since the biosimilar drugs are structurally indifferent from endogenous proteins. However, also biologicals can induce ADA formation. Interferon (IFN)-β, for example, is used as a treatment for multiple sclerosis. Neutralizing IFN-β-AB were detected and affected the efficacy of the treatment (Sorensen et al., 2003). ADA against biologicals bare the additional risk of cross-reacting with the endogenous protein potentially inactivating the endogenous pathway and leading to a sustained immune response with long-term impact on the signalling pathway (Table 1-1). Treatment with erythropoietin (EPO) led to anti-EPO-AB formation and was associated with pure-red cell aplasia (Casadevall et al., 2002). Similarly, thrombopoietin (THPO)-ADA as response to THPO-treatment led to thrombocytopenia caused by cross-reaction of THPO-ADA against endogenous THPO (Li et al., 2001). In some cases, the immune response is transient and diminishes over time. In patients with lumbar spinal fusion that were treated with recombinant human bone morphogenetic protein (rhBMP) 7, antibodies against BMP7 were found in 50% of patients but declined over time (Sauerborn et al., 2011).

Table 1-1: Anti-drug antibodies against biologicals.

Drug	**Consequence**	**Reference**
Interferon (IFN)-β	neutralizing IFN-β-AB reduced drug efficacy	(Sorensen et al., 2003)
Erythropoietin (EPO)	pure-red cell aplasia	(Casadevall et al., 2002)
Thrombopoietin (THPO)	thrombocytopenia	(Li et al., 2001)
Bone Morphogenetic Protein (BMP) 7	BMP7-AB	(Sauerborn et al., 2011)
Infliximab	anti-murine Ig-AB, loss of treatment response	(Steenholdt et al., 2013)

1.3 Bone

1.3.1 Bone formation during development

The skeleton has both structural and metabolic functions. Bone is a reservoir for minerals in particular calcium and phosphorous. Bone consists of 10% collagen, 65% hydroxyapatide (a salt of Ca and P), magnesium, sodium, bicarbonate and 25% water.

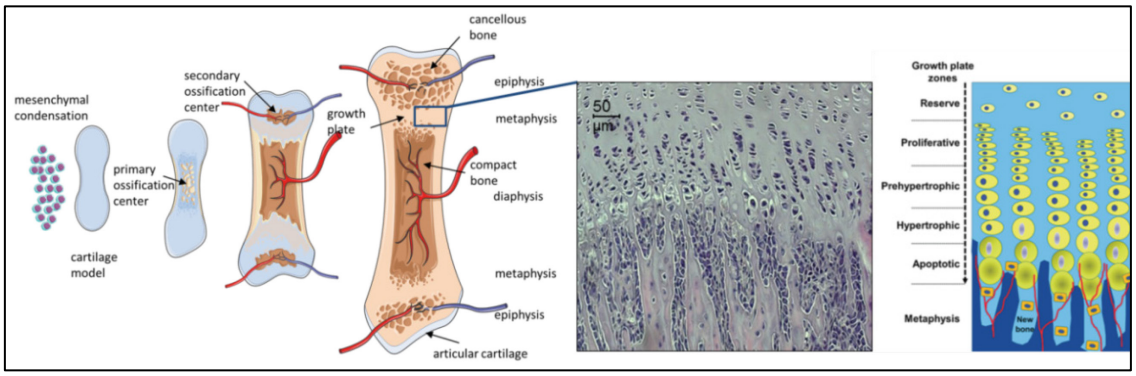

Figure 1-7: Endochondral ossification. Bone grows in length during development or during fracture healing starting from a cartilaginous model. Primary and secondary ossification centres form and the bone matrix is mineralized to compact bone. In the growth plate chondrocytes start to proliferate, increase in size and become hypertrophic, eventually they die and their secreted matrix is remodelled into new bone. As more and more bone is laid down the bone grows in length. Modified after (Williams, 2013) with parts of Servier Medical Art, histology picture kindly provided by Dr Nicole Pietschmann.

Endochondral Ossification is bone formation of the long bones arising from a cartilaginous template (Figure 1-7). Deriving from mesenchymal stem cells and being the only cells in cartilage, chondrocytes produce and maintain the cartilaginous matrix consisting of collagens and proteoglycans. Chondrocytes located in the growth plate proliferate, secrete collagen and line up in columns. Eventually they stop proliferating and become enlarged and hypertrophic. These hypertrophic chondrocytes secrete hormones and direct mineralization of cartilage between the columns. The cells undergo apoptosis and osteoblasts together with osteoclasts remodel the mineralized cartilage into bone (Lieberman, 2005).

Intramembranous ossification is bone formation of the flat bones like skull, mandible and clavicle, and also during natural healing of bone fractures. It is the formation of spongy bone through the direct conversion of undifferentiated mesenchymal cells into bone. This process does not involve cartilage and the mesenchyme provides the template (Lieberman, 2005).

1.3.2 Bone remodelling cycle

Adult bone undergoes constant remodelling which is a balance between bone resorption and bone formation (Figure 1-8). The anatomical microenvironment where bone remodelling occurs is a basic multicellular unit (BMU) and consists of bone-resorbing osteoclasts, bone-forming osteoblasts, osteocytes and lining cells covering the bone surface (Gonciulea and de Beur, 2015). Bone remodelling is under systemic and local control involving endocrine, paracrine, neural and mechanical stimuli (Hofbauer et al., 2014).

A network of osteocytes embedded in the bone matrix senses microdamage or mechanical loading. They produce sclerostin, Fibroblast Growth Factor 23 (FGF23) and Receptor Activator of NF-κB (RANK) ligand (RANK-L). Osteocytes can activate bone remodelling upon mechanical stress. They are aligned in osteoids and are in contact to each other through an interconnected canalicular system although separated by the mineral matrix.

The first step is activation during which osteoclasts are stimulated to differentiate. Osteoclasts resorb the bone thereby digesting the bone matrix which releases growth factors that stimulate preosteoblast attraction, proliferation and differentiation. Osteoclasts are bone resorbing cells derived from multipotent hematopoietic stem cells that secrete enzymes to digest organic matrix (acidic medium). They are generated by the fusion of hematopoietic stem cells into giant, multi-nucleated cells, a process called osteoclastogenesis (Lieberman, 2005; Sims and Martin, 2014). When macrophage colony-stimulating factor (M-CSF) is produced the cells can still become B-lymphocytes but the expression of c-fos and RANK marks the full commitment to the osteoclast lineage. RANK-L and M-CSF are two proteins essential and sufficient for osteoclast differentiation. The differentiation factor RANK-L is produced by osteoblasts, osteocytes and stromal cells and interacts with the receptor RANK expressed on the cell surface of osteoclast precursors (Danks and Takayanagi, 2013). RANK-L activity can be antagonized by the decoy receptor of the TNF receptor family osteoprotegerin (Opg) which is produced by osteoblasts. Accordingly, Opg-deficiency leads to osteoporosis (Gonciulea and de Beur, 2015). Then mononuclear cells initiate the transition from resorption to formation. Monocytes or macrophages remove bone debris.

Osteoblasts derived from mesenchymal stem cells lay down new bone which is mineralized. Runt-related transcription factor 2 (Runx2) and Osterix (Osx) are crucial factors in osteoblast differentiation. Mature osteoblasts produce new extracellular matrix rich in type 1 collagen. The majority of osteoblasts undergo apoptosis, the minority turns into lining cells or osteocytes.

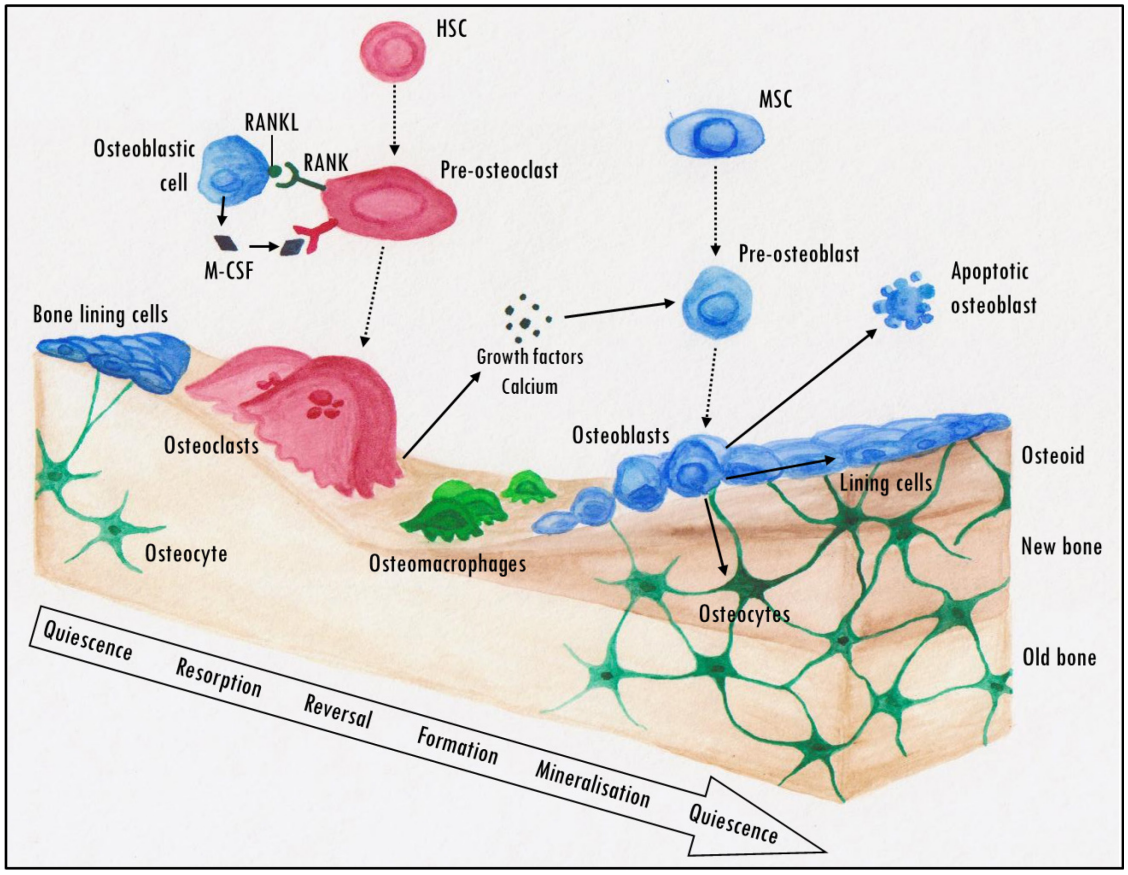

Figure 1-8: Bone remodelling cycle. Osteocytes embedded in the matrix sense microcracks. Osteoclasts, differentiated from hematopoietic stem cells, resorb the old bone by secreting digestive enzymes and providing an acidic milieu. Osteoblasts are activated to differentiate from mesenchymal stem cells and form the new bone. They die by apoptosis or become lining cells or osteocytes. The new bone matrix is mineralized. HSC: hematopoietic stem cell, MSC: mesenchymal stem cell, M-CSF: macrophage colony-stimulating factor, RANKL: Receptor Activator of NF-κB ligand. Figure produced by Gesa Nöhren.

1.3.3 Bone as an endocrine organ

For a long time it was thought that bone is simply a target of the endocrine system but today there is evidence that bone is a true endocrine organ in itself secreting the hormones FGF 23 and Osteocalcin. FGF23 is a phosphate-regulating hormone produced by osteoblasts and osteocytes. Klotho is the co-receptor for FGF23, and it is noteworthy that bone mineralization is impaired both in FGF-/- and in Klotho-/- mice (Murali, Roschger et al. 2015). Osteocalcin

is an osteoblast-derived hormone regulating energy and glucose homeostasis. It induces insulin secretion by pancreatic islet cells. This was shown by the co-cultivation of mouse osteoblast and mouse pancreatic islet cells leading to an increase in insulin production by the islet cells. This effect was not visible using Osteocalcin-/- osteoblasts (Wei and Karsenty 2015).

1.3.4 Fracture repair

Primary (direct) bone healing occurs when the bone fracture ends are aligned with minimal interfragmentary movement or pressed against each other. This is only possible for gaps less than 200-500 µm in size. Occurring without callus formation, the new bone is formed by the interplay of osteoclasts and osteoblasts in a manner similar to the process of the bone remodelling cycle (Lieberman, 2005).

Secondary (indirect) bone healing (= callus formation) occurs with larger defects or poor fixation. It goes along with hematoma formation and activation of the inflammatory cascade. A callus forms at the fracture gap. Then, in a process similar to that which takes place in the growth plates during bone development (endochondral bone formation), chondrocytes form cartilage which is then remodelled into bone by osteoblasts. A pseudarthrosis may develop in case the bone ends are not stabilized sufficiently by the callus (Lieberman, 2005).

1.3.5 Fracture treatment in clinical context – the diamond concept

After fracture, bone often completely regenerates to its original composition without the formation of a scar. It can therefore be considered as a truly regenerative tissue. Usually, a fracture gap is closed within 3-6 months after trauma. However, some fractures (approx. 10%) show healing difficulties leading to delayed healing, or a non-union also known as pseudarthrosis. There are a number of parameters affecting the healing process including the severity of the initial insult as well as age and health of the patient (Moghaddam et al., 2010; Moghaddam et al., 2011; Claes et al., 2012).

A compromised healing situation requires an intervention to reactivate and enhance natural bone formation. The major aspects that need to be considered are osteogenic cells, osteoconductive scaffolds, osteoinductive stimulants (hormones and local growth factors) and the mechanical environment,

summarized as the diamond concept (Giannoudis et al., 2007) which was extended by the aspect of vascularity (Giannoudis et al., 2008) (Figure 1-9). Interventions according to the diamond concept involve an assessment of all of these aspects for a given patient and the attempt to optimize the therapeutic measures resulting in an individualized therapy plan. Treatment of non-unions following this concept proved to be a reasonable and successful strategy (Moghaddam et al., 2015; Schmidmaier and Moghaddam, 2015; Miska et al., 2016). In the early stage the therapeutic treatments in delayed union cases may include biophysical stimulation, e.g. full weight bearing, low-intensity pulsed ultrasound, shockwave or electromagnetic field stimulation. Biological enhancement of bone regeneration is the basis for treatment of non-unions with autologous cancellous bone graft being considered the gold standard in the surgical treatment of non-unions, but the limited availability is problematic (Gregersen, 1990). Amongst the locally applied biological enhancers are calcium phosphate or collagen sponges as osteoconductive material, growth factors like EPO, FGFs or BMPs as osteoinductive agents and synthetic polymers or autologous bone as osteogenic material (Einhorn and Gerstenfeld, 2015).

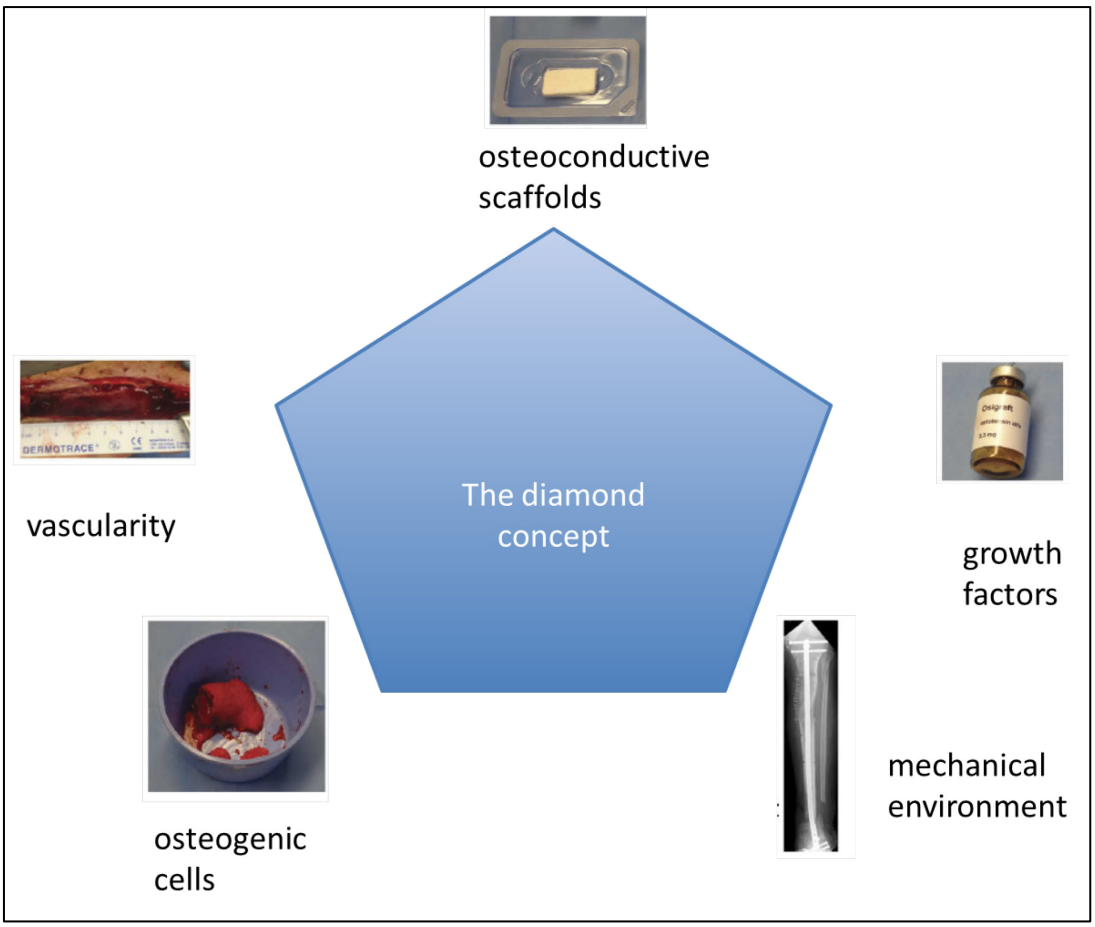

Figure 1-9: Fracture treatment according to the diamond concept. In difficult fracture situations or compromised healing situations, the aspects osteoconductive scaffolds, vascularity, osteogenic cells, growth factor status and mechanical environment are analysed for each individual patient and a combination therapy is generated to improve all of these aspects in a personalized manner. Modified after (Moghaddam et al., 2015).

1.4 Insulin and Insulin-like growth factor 1 as important growth factors in bone

Insulin-like growth factor 1 (IGF1) and insulin are endocrine signals for anabolic reactions. IGF1 circulates in blood in mircromolar concentrations. The majority of the hormone is bound to IGF binding proteins (IGFBPs) while insulin circulates freely in the blood at nanomolar concentrations. It is believed that IGF1 becomes active and is released from its binding proteins in close proximity to its receptor. The major functional difference between IGF1 and insulin is their tissue-specific expression and target cell pattern. But nevertheless, their tyrosine-kinase-linked receptors are expressed in almost all tissues (LeRoith and Yakar, 2007). IGF1 receptor (IGF1R) binds IGF1 with high affinity and insulin with low affinity. Conversely, insulin receptor (IR) binds insulin with high

affinity and IGF1 with low affinity. IGF1R and IR can form heterodimers that bind both ligands.

To study IGF1 function in more detail various tissue-specific knockout systems have been used. A liver-specific *igf1* gene-deletion mouse model (LID) was established by using the Cre/loxP recombination system (Yakar et al., 1999). It was expected that this *igf1* gene knockout in the liver would completely remove circulating Igf1. However, circulating Igf1 was only reduced by 75%. The phenotype was normal growth and development which let the authors to conclude that the paracrine/autocrine form of Igf1 is more important for growth and development than the hepatically-derived circulating hormone.

Further, mice lacking the Igf1r in osteoblasts were created by using the Cre/loxP system. Normal to elevated numbers of osteoblasts were detected but the rate of bone formation was reduced, cancellous bone volume was reduced, and the trabecular structure was altered. Surprisingly, osteoblasts and osteoclasts were hyperactive. These findings hint towards an important role of Igf1 in coupling matrix biosynthesis and sustained mineralization (Zhang et al., 2002).

Mice lacking the osteoblast IR were generated by Cre-expression under the osteocalcin-promoter and recombination of the *IR* gene (Fulzele et al., 2010). IR signalling in osteoblasts is involved in osteoblast development and bone formation. This study showed the failure of osteoblast maturation and decreased bone formation. The mice developed adiposity and insulin resistance (Fulzele et al., 2010).

Mice overexpressing Igf1 in osteoblasts showed no change in circulating Igf1-levels and in body growth (Zhao et al., 2000). An increased bone formation rate, greater bone mineral density and increased cancellous bone volume was observed, but with no change in number of osteoblasts or osteoclasts.

Insulin and bone metabolisms are linked via energy metabolism. Osteoblasts express the IR, with insulin signalling leading to bone formation. Insulin signalling in osteoblasts also regulates the production and bioavailability of the hormone osteocalcin (Wei et al., 2014) which itself regulates the insulin secretion in the pancreas (Fulzele et al., 2010). Therefore, insulin and osteocalcin create a bone-pancreas endocrine loop. Insulin is one of the players connecting the endocrine organ bone to other endocrine organs.

1.5 Bone morphogenetic proteins as important growth factors in bone

Groundwork for the discovery of bone morphogenetic proteins (BMP) was laid in 1965 by Marshall Urist and his team when they demineralized bone, implanted it in skeletal muscle and observed new ectopic bone formation. It was shown for the first time that demineralized bone extract itself was able to induce bone formation (Urist, 1965). The proof that these osteogenic factors are indeed proteins came nearly 20 years later by Hari Reddi (Sampath and Reddi, 1981) and soon the DNA of these proteins was isolated and the proteins expressed (Wozney et al., 1988).

BMP are pleiotropic cytokines that are involved in cell proliferation, survival/apoptosis, differentiation and migration. They belong to the transforming growth factor (TGF) β superfamily together with the TGFs, activins and inhibins and growth-and-differentiation factor (GDF), with over 20 members having been identified. BMP carry six conserved cysteine residues that form a cysteine-knot motif through internal disulphide bonds, and a seventh cysteine residue that forms a disulphide bond with a second polypeptide to form a homodimer. BMP are produced as pre-pro-proteins. The N-terminal pre-domain (signal peptide) directs the polypeptide to the secretory pathway. The rather large pro-domain is important for proper folding (Horbelt et al., 2012; Carreira et al., 2014).

The ligands bind as dimers to serine/threonine kinase receptors. Two classes of receptors are required for signal transduction. In humans seven type I receptors termed activin-like receptor kinase (ALK) 1-7 and five type II receptors (TGFβRII, ActR-IIa, ActR-IIb, AMHR-II and BMPR-II) have been identified. There is ligand-receptor promiscuity. This means one ligand binds to several receptors adding to the complexity of BMP signalling (Mueller and Nickel, 2012). The type I receptors contain a glycine and serine rich domain (GS domain) located between the transmembrane and kinase domains. The type II receptor´s serine/threonine kinase is constitutively active.

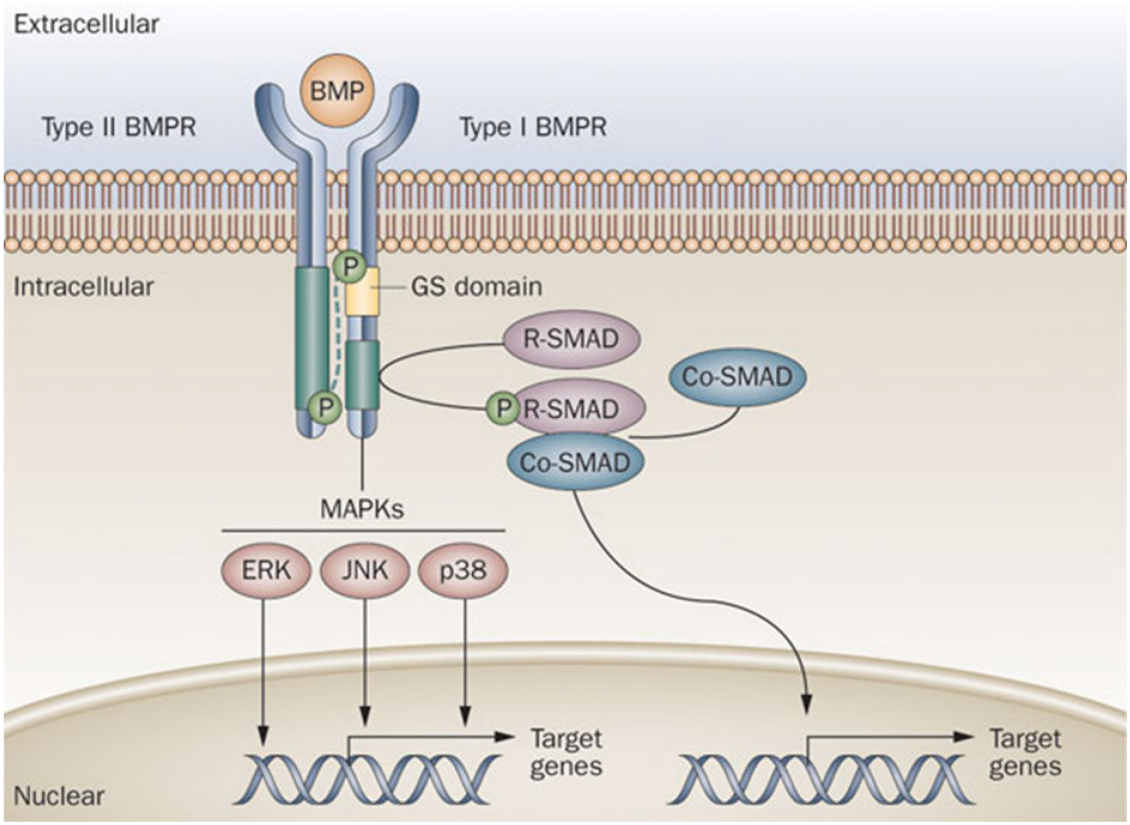

Figure 1-10: BMP signal transduction. BMPs bind as dimers either to a pre-formed complex of type I and II receptors initiating signal transduction via the SMAD pathway or bind to type I receptor followed by recruitment of type II receptor and signalling through non-SMAD pathway (Sieber et al., 2009; Shore and Kaplan, 2010).

BMP signalling is transmitted via the SMAD-pathway (Figure 1-10). Upon ligand binding and hetero-oligomerization of type I and II receptor dimers, the type II receptor phosphorylates the GS-domain of the type I receptor. Type I passes the signal on to SMAD proteins by phosphorylating receptor-related (R) Smad (SMAD 2/3 for TGFs and SMAD 1/5/8 for BMPs). Phosphorylation leads to dimerization of R-SMADs and association with a common mediator (co)-SMAD (SMAD4). This heterotrimer is translocated into the nucleus, associates with transcription factors and transcription of target genes is initiated. The activation of R-SMAD can be inhibited through inhibitory (I)-SMAD. Next to SMAD signalling, BMP can also signal via non-SMAD pathways e.g. MAPK/p38 signalling. Two ways of receptor assembly have been described. The first possibility is a pre-formed complex (PFC) of pre-assembled BMP type I and type II receptors. Ligand binding to the pre-assembled complex would then directly initiate the SMAD pathway. The second option is the assembly of a

BMP-induced signalling complex (BISC). Hereby, the BMP-bound type I receptor would recruit the type II receptor leading to signalling through a non-SMAD pathway (Sieber et al., 2009).

Regulation of BMP signalling is highly complex and the realization of specificity is not completely understood. What makes the controlled signalling so special and hard to understand is the fact of receptor promiscuity, the ability of BMP to form heterodimers and the shared components like SMAD4. All of these aspects seem to be part of the tight regulation in BMP signalling. Nevertheless, the tight regulation of BMP signals starts with the well-controlled ligand production and control of ligand bioavailability. The secreted antagonists like Chordin, Gremlin and Noggin are able to interfere with BMP signalling by forming inactive complexes, and the ligands interact with a number of extracellular matrix components as well as membrane-bound effectors thereby attenuating their signal transmission. Pseudoreceptors and Co-receptors may further inhibit signalling. The receptors are regulated in their ability to oligomerize at the plasma membrane, for which lateral diffusion through the membrane is necessary. Receptor presence on the cell surface is regulated by receptor endocytosis. The regulation of SMAD activity further regulates the intracellular signal (Kopf et al., 2014).

1.6 Hypothesis

Bone formation during fracture healing is a highly complex process involving numerous hormones and growth factors including IGF1, insulin and several BMPs (Zaidi, 2007). The effects of these growth factors are mediated by their receptors present in the cell membrane of e.g. lymphocytes, osteoblasts or stem cells. A considerable number of fracture patients display disturbed or delayed bone healing. As BMP7 is involved in bone anabolism, recombinant BMP7 can be applied to treat some of these difficult healing situations. Antagonizing autoantibodies (aAB) against the IGF1-receptor have been identified in about 10% of human sera (Minich et al., 2012). These could be involved in disturbed healing. Therefore, the objective of this thesis is to test whether IGF1R-aAB are present in fracture patients and related to fracture healing. And further, are other aAB against growth factors present in the fracture patients, or become induced upon treatment by therapeutic growth factors? The following specific research questions are addressed.

Can autoantibodies against bone-related growth factors and their receptors be identified in sera of fracture patients, are they induced by recombinant growth factor treatment and if so, are they related to therapy outcome or side-effects?

To test these issues a cohort of sera from fracture patients with different treatments were available. Novel detection assays for IGF1-, insulin- and BMP-aAB were developed. The following working hypotheses were tested:

1. IGF1R- and IR-aAB can be detected in sera of fracture patients and are more prevalent in a cohort of fracture patients than in other cohorts
2. IGF1- and insulin-aAB can be detected in human serum of healthy subjects and diabetes patients and are associated with disturbed bone healing
3. Natural BMP-aAB can be detected in sera of healthy subjects and are more prevalent in sera of fracture patients than in control subjects.
4. BMP7-aAB are induced by rhBMP7-therapy
5. BMP-aAB interfere with BMP signal transduction.

2 Material and Methods

Chemicals were purchased in *pro analysi*-quality by the following providers if not stated otherwise; Carl Roth (Karlsruhe), Merck (Darmstadt), Roche Diagnostik (Mannheim), Serva Feinbiochemika (Heidelberg) und Sigma-Aldrich Chemie (Steinheim).

Standard laboratory equipment was purchased by Amersham (München), A. Hartenstein (Würzburg), Bio-Rad Laboratories (München), GE Healthcare (München), Invitrogen (Darmstadt), Merck Biosciences (Schwalbach), Eppendorf (Hamburg), peqLab Biotechnologie (Erlangen), Sarstedt (Nümbrecht), Sigma-Aldrich Chemie (Steinheim), Biochrom (Berlin), Life Technologies (Darmstadt), VWR (Darmstadt) and Qiagen (Hilden).

If not stated otherwise, equipment and materials were purchased from companies located in Germany.

Table 2-1: Software and database used to analyse the data.

Software/database	**Supplier**
Endnote X7	Thomson Reuters, New York, USA
GraphPad Prism version 5.01	GraphPad Software, San Diego, USA
IBM SPSS Statistics Version 19	IBM Deutschland GmbH, Ehningen
Microsoft Office 2010	Microsoft, Unterschleißheim
PubMed	http://www.ncbi.nlm.nih.gov/pubmed
Servier Medical Art	http://www.servier.com/Powerpoint-image-bank

Table 2-2: Laboratory equipment.

Device	**Supplier**
Analytical balance A120S	Sartorius, Göttingen
Autoklav varioklav	H+P Labortechnik, Oberschleißheim
Centrifuge Heraeus Megafuge 1.0R	Heareus Sepatec GmbH, Hanau
Chain luminometer Autolumat Plus LB 953	Berthold technologies, Bad Wildbad
CO_2-Incubator (Heracell)	Heraeus Sepatec GmbH, Hanau
Electrophoresis chamber (horizontal)	Roth, Karlsruhe
Gel documentation system alphaimager®	Alpha Innotec, San Leandro, USA
Magnetic stirrer model L32A	Hartenstein,Würzburg
Microscope axioskop 2	Carl Zeiss, Oberkochen
Neubauer cell counting chamber	Roth, Karlsruhe
PCR-Cycler Primus 25 und 96plus	MWG Biotech, Ebersberg
pH-Meter inolab Benchtop	WTW, Weilheim
Pipettes 0.5-10µl, 10-100µl, 100-1000µl	Eppendorf, Hamburg
Plate luminometer Mithras LB940	Berthold technologies, Bad Wildbad
Plate shaker Titramax 1000	Heidolph Instruments, Schwabach
Spectrophotometer Nanodrop 1000	PEQLAB GmbH, Erlangen
Sterile bench Model 1.2 (herasafe)	Heraeus Sepatech GmbH, Hanau
Thermomixer comfort	Eppendorf, Hamburg
Trans-blot® semi-dry transfer cell	Bio-Rad Laboratories, München
UV-Transilluminator , V-90M	Benda, Weisloch
Vortex	Eppendorf, Hamburg

2.1 Molecular Biology

2.1.1 Insulin and IGF1 DNA sequences

Table 2-3: Summary of the plasmids and their sources.

Plasmids	Origin/reference
pIRESneo	Clontech (Palo Alto, California)
pIRES-LUC	kindly provided by Dr Waldemar Minich (Minich et al., 2012)
pIRES-SEAP	kindly provided by Dr Waldemar Minich [unpublished]
pIRES-IGF1-LUC	cloned during this work
pIRES-IGF1-SEAP	cloned during this work
pIRES-insulin-LUC	cloned during this work
pIRES-insulin-SEAP	cloned during this work

The *IGF1* and *insulin* gene sequences were amplified and purchased from Life technologies (Carlsbad, USA). The sequences and amount of DNA provided are depicted in Table 2-4. In order to prevent proteolytic processing from proinsulin to insulin, the basic cleavage sites were mutated at the genetic level, resulting in the integration of different amino acids during synthesis. Both sequences were extended providing suitable restriction sites. The delivered DNA was re-suspended in pure water with restriction enzyme digestion being performed as described in section 2.1.2. The fragments were resolved via agarose gel electrophoresis (section 2.1.3), and extracted (section 2.1.4). The DNA fragments were ligated into the vectors pIRES-SEAP and pIRES-LUC (section 2.1.5) and subsequently transformed into *E.coli* (section 2.1.6). Positive clones were identified and the plasmids produced in high quantities.

Table 2-4: *Insulin* and *IGF1* DNA sequences.

insulin
1000 ng 354 bp sense strand, 5' - 3' GCCACCATCGATATCATGGCCCTGTGGATGCGGCTGCTGCCCCTGCTGGCTCTG CTGGCACTGTGGGGACCTGATCCTGCCGCCGCTTTCGTGAACCAGCACCTGTGT GGCAGCCACCTGGTGGAAGCCCTGTATCTCGTGTGCGGCGAGCGGGGCTTCTTC TACACCCCTAAGACCTGCTGGGAGGCCGAGGATCTGCAAGTGGGCCAGGTGGAA CTGGGCGGAGGACCTGGCGCTGGATCTCTGCAGCCTCTGGCCCTGGAAGGCAG CCTGCAGGAATGCGGCATCGTGGAACAGTGCTGCACCAGCATCTGCTCCCTGTA CCAGCTGGAAAACTACTGCAACGAATTCATC Arg-Arg → Cys-Trp Arg-Lys → Glu-Arg
IGF1
550 ng 372 bp ATCGATATCATGGGCAAGATCAGCAGCCTGCCCACCCAGCTGTTCAAGTG CTGCTTCTGCGACTTCCTGAAAGTGAAGATGCACACCATGAGCAGCAGCC ACCTGTTCTACCTGGCCCTGTGCCTGCTGACCTTCACCAGCTCTGCCACA GCCGGCCCTGAGACACTGTGTGGCGCTGAACTGGTGGACGCCCTGCAGT TCGTGTGCGGCGACAGAGGCTTCTACTTCAACAAGCCCACCGGCTACGGC AGCAGCTCCAGAAGGGCTCCTCAGACCGGCATCGTGGACGAGTGCTGTTT CAGAAGCTGCGACCTGCGGCGGCTGGAAATGTACTGCGCCCCTCTGAAG CCTGCCAAGAGCGCCGAATTCATC

2.1.2 Restriction endonuclease digestion

Restriction digestion was usually performed in a total volume of 20 µl. A typical reaction included 500 ng to 1 µg DNA in presence of 1 unit of each restriction enzyme. Restriction enzymes were obtained from New England Biolabs (NEB). Appropriate digestion buffers and potential additives were added as specified by the restriction enzyme used and were also obtained from NEB. When multi-enzyme digestions were performed, the digestion buffer offering the highest activity for all enzymes applicable to that particular reaction was chosen. Digestions were incubated at 37°C for 1 hour. For separation of digestion products, agarose gel electrophoresis (section 2.1.3) and DNA isolation by gel extraction (section 2.1.4) were performed.

Analytical restriction enzyme digestion was used to check the correct integration of an insert into a vector. Again, typically 500 ng to 1 µg of a plasmid mini preparation purified DNA was digested in 20 µl reaction volumes containing appropriate reaction buffers at 37°C for 1 hour, before being resolved via

agarose gel electrophoresis (section 2.1.3). Restriction enzymes were chosen that yielded DNA fragments demonstrating a specific post-electrophoretic band pattern, thus enabling the clear distinction between plasmids containing DNA fragments from those that did not.

2.1.3 Agarose gel electrophoresis

Agarose gels were prepared by mixing agarose into 1x TAE buffer (1x TAE buffer dilution of 50x TAE buffer with dH_2O; 50 x TAE buffer 242 g Tris, 57.1 mL acetic acid, 18.5 g Na_2EDTA x $2H_2O$, dH_2O to 1 L, pH 7.5) at 1-2% concentration, dependent upon the size of the products to be resolved. The DNA was visualised under UV-light via the intercalator Ethidium Bromide. DNA samples were mixed with 6x DNA sample buffer (0.25% (w/v) Bromphenol blue, 30% (w/v) glycerol in dH_2O) and loaded into the loading pockets of the gel. The electrophoresis chamber was filled with 1x TAE buffer. The DNA fragments were usually separated at 120 Volt for 1 hour or until the bands were clearly separated. A DNA mix of previous defined lengths (1 kb gene ruler, Fermentas, Burlington) was always included to estimate the sizes of the DNA. Documentation was done using the Gel documentation system alphaimager.

2.1.4 Gel extraction and purification of DNA fragments

For extraction of DNA fragments from an agarose gel the Perfectprep® Gel Cleanup Kit (Eppendorf, Hamburg) was used according to the manufacturer's instructions. In brief, DNA fragments were visualized in the agarose gel using a UV-transilluminator, excised with a scalpel and transferred to a clean micro centrifuge tube. For each 100 mg of agarose gel, 300 µl GelCleanUp buffer was added and incubated at 50°C for 10 min until gel slices were dissolved. The solution was loaded onto the spin column. DNA bound to the silica membrane of the column was washed with washing buffer and eluted with 50 µl water.

2.1.5 Ligation of DNA fragments

For ligation reactions T4 DNA ligase 400,000 U/mL was used (NEB). The ligase catalyses the ATP dependent formation of a phosphodiester bond between a 5' phosphate and a 3' hydroxyl terminus in double stranded DNA with cohesive or

blunt ends. For ligation of insert and plasmid DNA an at least five fold molar excess of insert compared to vector DNA was used in the presence of 400 units of T4 DNA ligase in T4 ligase buffer with 10 mM ATP 10x (NEB). A typical ligation reaction is depicted in Table 2-5. Ligation reactions were incubated for 1 hour at RT. Ligation samples were used to transform chemically competent *E.coli* via heat-shock (section 2.1.6).

Table 2-5: Ligation reaction both with insulin and IGF1 as fragments.

SEAP		luciferase	
6 µl	fragment (~6 ng/µl)	12 µl	fragment (~6 ng/µl)
11 µl	pIRES-SEAP (18 ng/µl)	5 µl	pIRES-LUC (190 ng/µl)
2 µl	T4 buffer	2 µl	T4 buffer
1 µl	T4 ligase (400 kU/mL)	1 µl	T4 ligase
20 µl		20 µl	

2.1.6 Transformation of E.coli

Competent *E.coli* DH5α (Life Technologies, Darmstadt) were kept on ice while 5 µl of the ligation reaction was added. The mixture was incubated for 30 minutes on ice and then DNA transformation was performed by heat shock (45 seconds at 42°C followed by 2 minutes on ice). 500 µl LB-medium (Table 2-6) was added and the bacteria were incubated for 1 hour at 37°C shaking. The bacteria were plated on LB-agar (Table 2-6) plates containing 100 µg/ml ampicillin (Sigma Aldrich, München). The plates were incubated over night until single clones were clearly visible. Clones were selected, plasmids isolated by use of FastPasmid® Mini Kit (Eppendorf, Hamburg) and positive clones were identified by restriction digestion leading to characteristic band pattern in gel electrophoresis as described in section 2.1.2. Potentially positive clones containing the proper plasmids were selected and amplified by maxi preparation (Pure Yield™ Plasmid Midiprep System, Promega, Mannheim). The purified plasmid was verified by sequencing.

Table 2-6: Media for bacterial cell culture.

LB-Agar (Lennox), Roth		LB-medium (Lennox), Roth	
Trypton	10 g/l	Trypton	10 g/l
Yeast extract	5 g/l	Yeast extract	5 g/l
NaCl	5 g/l	NaCl	5 g/l
Agar	15 g/l		
35 g in 1l → autoclaved		20 g in 1l → autoclaved	

2.2 SDS-PAGE and Western Blot

Proteins were separated according to their molecular weight via **s**odium **d**odecyl **s**ulphate-**p**oly**a**crylamide **g**el **e**lectrophoresis (SDS-PAGE). Electrophoretic mobility of the proteins is accomplished in an electric field based on protein-linearization and overall net negative charge imparted by the detergent SDS. The protein samples were mixed with mercaptoethanol-containing 4x SDS loading buffer and denatured for 10 minutes at 95°C. The SDS-PAGE was started at 80 Volt for the stacking gel and continued at 120 Volt for the separation gel. A protein ladder (PageRuler™ Prestained Protein Ladder 10-25 kDa, ThermoScientific) was utilised to identify the molecular weight of the protein bands. All reagents and recipes are listed in Table 2-7.

In a second step the Western Blot was performed. The proteins were transferred from the gel to a nitrocellulose membrane (GE Healthcare) by the semi-dry blotting method at 20 Volt and 250 mA for 30 minutes in 1x transfer buffer. After blotting the membrane was stained by Ponceau S to check the blotting efficiency and to prove equal loading of the lanes. The membrane was then incubated for 15 minutes in blocking solution (5% skim milk powder in 1xTBST) followed by three washing steps for 10 minutes in TBST. The primary antibody was diluted in blocking solution and applied over night at 4°C. All antibodies are listed in Table 2-8. The next day, the membrane was washed three times for 10 minutes in TBST and incubated for one hour in diluted secondary antibody followed by three washing steps for 10 minutes in TBST. The protein bands were finally detected by enhanced chemiluminescence (ECL™ Western Blotting Detection-Kit, GE Healthcare) according to the manufacturer's instructions.

Table 2-7: Reagents, buffers and recipes used to perform SDS-PAGE and Western Blot.

Solution	**Composition**	
20% gel solution	162.3 ml 30% Acrylamide, 65 ml 2% Bisacrylamide,→ dH_2O to 150 ml	
4x SDS loading buffer (Laemmli)	200 mM Tris pH 6.8, 40% Glycerol, 16% SDS, 20% 2-mercaptoethanol, 0.02% Bromphenolblue → dH_2O to 10 ml	
separation gel buffer	1.5M Tris-HCL pH8.8	
stacking gel buffer	0.5 M Tris-HCL pH 6.8 with Bromphenolblue	
1x SDS running buffer	192 mM Glycine, 25 mM Tris, 0.1% SDS	
1x transfer buffer	25 mM Tris, 150 mM Glycine, 10% methanol	
blocking solution	5% skim milk powder in 1xTBST	
1x TBST	25 mM Tris, 125 mM NaCl, 0.3 % Tween, pH adjusted to 8.0	
20x Ponceau S staining buffer	10 g Ponceau S, 50 ml acetic acid, → dH_2O to 500 ml	
1x Ponceau S staining buffer	25 ml 20x Ponceau S staining buffer, 25 ml acetic acid, → dH_2O to 500 ml	
SDS-PAGE	**Separation gel (12.5%)**	**Stacking gel**
dH_2O	1.25 ml	2 ml
separation gel buffer	2.5 ml	-
stacking gel buffer	-	1 ml
20% gel solution	6.25 ml	1 ml
10% APS	100 µl	40 µl
TEMED	10 µl	4 µl

Table 2-8: Primary and secondary antibodies used for Western Blot.

Antibody	**applied dilution in Western Blot**	**Supplier**	**Article number**
primary antibodies			
anti-human proinsulin monoclonal (mouse)	1:1000	Abcam , Cambridge, UK	ab1965
anti-human IGF1 monoclonal (mouse)	1:5000	Abcam, Cambridge, UK	ab40789
polyclonal anti human BMP7-AK (goat)	1:2500	Santa Cruz	sc-6899
anti-human BMP2 polyclonal (rabbit)	1:2500	Dianova, Hamburg	CYT-26591
anti-insulin polyclonal (guinea pig)	1:1000	Abcam , Cambridge, UK	ab 7842
anti-LUC polyclonal (goat)	1:2000	Promega, Mannheim	G7451
secondary antibodies			
anti-rabbit IgG-HRP (goat)	1:2000	Dako, Hamburg	P0448
anti-mouse IgG-HRP (sheep)	1:2500	GE Healthcare, Pollard Wood, UK	LNXA931
anti-sheep IgG-HRP (rabbit)	1:1000	Dako, Denmark	P0163
anti-goat IgG-HRP (rabbit)	1:2000	Dako, Hamburg	P0449
anti-guinea pig IgG-HRP (rabbit)	1:1000	Dako, Denmark	P0141

2.3 Eukaryotic cell culture

Eukaryotic cell lines were cultured in a humidified incubator at 37°C with 5% CO_2. Usually, cells were cultured in T75 (75 cm^2) flasks or p60 (diameter 20 cm) culture dishes. All cell lines are summarized in Table 2-9, reagents in Table 2-10 and all media compositions in Table 2-11. All cell lines were cultured in DMEM/F12 supplemented with 10% FBS, henceforth referred to as Culture Medium.

Table 2-9: Cell lines and the cell banks from which they were sourced.

Cell line	Supplier
HepG2	DSMZ-No. ACC180
HEK293	ATCC® CRL-15733™
NIH3T3	ATCC® CRL-1658™

Table 2-10: Reagents used for cell culture experiments.

Cell culture supplements	Supplier
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich, St. Louis, USA
Dulbecco`s MEM (DMEM)	Biochrom, Berlin
Dulbecco`s MEM (DMEM)/F12 with $NaHCO_3$, L-Gln, 4.5 g/L D-Gluc	Life technologies, Carlsbad, USA
Foetal bovine serum (FBS)	Biochrom, Berlin
Geneticin (G418) sulphate, sterile filtered	Calbiochem/Merck-Millipore, Billerica, USA
PEI 40kDa	InVivo, Henningsdorf
Puromycin	InVivoGen, Toulouse, France
Trypsin	Life technologies, Carlsbad, USA

Table 2-11: Composition of different media used in cell culture experiments.

Cell culture media	Composition
Culture medium	DMEM/F12, 10% FBS
Freezing medium	40% DMEM/F12, 50% FBS, 10% DMSO
BSA-medium	DMEM/F12, 1% BSA, sterile filtered
G418-medium	DMEM/F12, 10% FBS, 0.8 mg/ml G418
DMEM	without FBS, without F12, without phenol red

2.3.1 Thawing of cells

Cryoconserved cell stocks were quickly thawed in a 37°C water bath. The cell pellet was then resuspended and added to 10 ml culture medium in a T75 cell culture flask. The next day, the medium was exchanged for fresh medium.

2.3.2 Culturing of cells

Cells were cultured in 10 ml culture media (DMEM/F12, 10% FBS) in T75 flasks. When cultures reached about 80-90% confluency, the cells were passaged at a ratio of 1:3 or 1:5 depending on the particular growth of the cell line. To this end, the old medium was aspirated and the cell layer was washed once with 5 ml 1x PBS. To detach the cells 1 ml Trypsin was added and the cells incubated for 5 minutes at 37°C. The trypsin digestion was stopped by adding at least 5 ml FBS-containing medium and cells were transferred to a new flask with fresh medium. Cells under passage number 40 were used for experiments.

2.3.3 Cryoconservation of cells

Cells were cultivated in T75 flasks until they reached 80% confluency, which is when they are in their exponential growth phase. Cells were trypsinized and centrifuged at 1000 rpm for 5 minutes at 4°C. The supernatant was discarded and the cell pellet resuspended in 3.5 ml freezing medium (Table 2-11). Cells from one T75 flask were divided into three cryo vials. Vials were kept on ice for 30 minutes, then stored at -80°C overnight before being transferred to liquid nitrogen, thus ensuring freezing (best 1°C per hour).

2.3.4 Transfection of cells for protein production

HEK293 cells were seeded in a 6-well plate at a cell number of 0.5*10^6 cells per well. After 24 hours of incubation at 37°C and 5% CO_2 the medium was exchanged for fresh culture medium. The transfection reaction was prepared (2 µg DNA in 100 µl DMEM + 6 µl PEI40kDa), vortexed and incubated for 15 minutes at RT. Finally, the transfection reaction was added to the cells. After two days the culture medium was exchanged for G418-medium for selection.

2.4 Expression of recombinant proteins IGF1-LUC, IGF1-SEAP, insulin-LUC and insulin-SEAP

The recombinant antigen-luciferase fusion proteins for autoantibody assays were produced in HEK293 cells (section 2.3.4). To this end, HEK293 cells were transfected in 6 well plates with the pIRES-IGF1-LUC, pIRES-IGF1-SEAP,

pIRES-insulin-LUC or pIRES-insulin-SEAP constructs (section 2.1.1) by the use of the transfection reagent PEI40kDa as described in section 2.3.4. Successfully transfected cells were selected by G418 resistance by cultivating with DMEM/F12, 10% FBS, 0.5-0.8 mg/ml G418. Transfected cells were expanded. For the SEAP fusion proteins, supernatants were collected. For the luciferase fusion proteins, cell extracts were prepared (section 2.4).

2.5 Supernatant and extract preparation of recombinant fusion reporter proteins

For supernatant preparation, transfected HEK293 cells were cultivated until 60 % confluent in a 75 cm^2 flask and then the medium was exchanged for 10 ml BSA-medium. After cultivation for two additional days, the supernatant was collected and glycerol added to a final concentration of 30%. The activity of the recombinant proteins was measured by mixing 10 µl supernatant with 200 µl 1:5 diluted SEAP substrate in SEAP buffer followed by an incubation of 20 minutes and 10 seconds measurement of RLU in the chain luminometer. Aliquots of the supernatant were prepared and stored at -80°C until further use.

For extract preparation, transfected HEK293 cells were expanded in p60 culture dishes (diameter 20 cm) and were cultivated in DMEM/10%FBS until maximum confluency was reached. Cells were scraped with a sterile cell scraper and transferred into falcon tubes (50 ml) on ice. The cell suspension was centrifuged at 3500 rpm for 5 minutes at 4°C, the supernatant discarded and 0.5 ml lysis buffer (50 mM Tris, 100 mM NaCl, 10% glycerol) added per cell culture dish. After gently vortexing, Triton X-100 was added to a final concentration of 2%, followed by incubation on ice for 15 minutes. The lysed cells were then centrifuged at 3500 rpm for 15 minutes at 4°C, the supernatant collected and aliquots of 1 ml stored at -80°C until further use. For activity measurement 10 µl extract was mixed with 300 µl luciferase substrate and measured for 10 seconds in the chain luminometer.

2.6 MACN-labelling of proteins

For the BMP7- and BMP2 autoantibody assay, reporter fusion proteins were not expressed as recombinant proteins. For this assay, the recombinant human proteins that are used clinically as treatment were labelled with the

chemiluminescence tag acridiniumester-N-hydroxy-succinimid (MACN, InVent Diagnostica GmbH). To this end, 0.1 mg of collagen-free rhBMP7 (Olympus Biotech) or rhBMP2 (Metronic) were labelled with MACN in an amine-free buffer. After incubating for 15 minutes in the dark, the labelling reaction was stopped by adding 1M Tris, pH 7.5. The MACN-labelled rhBMP7 was diluted in buffer (PBS, 1% BSA, 0.1% NaN_3) and separated from unbound MACN by using 10kDa MWCO centrifugal filter units (Centricon Ultracel-10K, Millipore, Eschborn). The labelled protein was stored in aliquots at -80°C.

Recombinant human Insulin-like growth factor 1 (rhIGF1) (QED Bioscience, San Diego, USA) and recombinant human insulin (rhinsulin) (Sigma Aldrich, München) were labelled with MACN following the same protocol and used in the IGF1 and insulin aAB assays.

2.7 Isolation of IgGs

Total IgG of aAB positive and negative sera were isolated by precipitation with Protein A. Serum samples (300 µl) were incubated with 600 µl of 50% protein A slurry in PBS (PorosA®, Applied Biosystems) and incubated under constant agitation overnight at 4°C. The supernatants were discarded and the pellets were washed six times with PBS. Precipitated IgG were eluted with 25 mM citric acid, pH 2.2. Seven fractions (500 µl each) were collected and neutralized by addition of 1M HEPES, pH 8.0. The volume of the eluate was reduced by spinning in centrifugal filtration units with a 50 kDa cut-off (Centricon YM-50, Amicon, Millipore), adjusted to a volume of 300 µl and stored at 4°C until use. The protein concentration was determined by Nanodrop.

2.8 Autoantibody precipitation assay

The principle of the autoantibody assay is based on the binding of autoantibodies to antigen fused to a reporter (luciferase, SEAP or MACN) followed by a precipitation of this antibody-antigen-reporter complex by protein A.

Antigen-luciferase extracts and antigen-SEAP supernatants were diluted 1:10 with binding buffer. Antigen-MACN was diluted 1:500 with binding buffer. 100 µl of diluted antigen-reporter was then incubated with 10 µl serum or adequate controls over night at 4°C. Per sample, 50 µl 10% Poros A were added and

incubated for 1 hour at RT on a shaker. The samples were washed with 1 ml washing buffer, centrifuged (5 minutes, 3500 rpm, 20°C) and the supernatant was aspirated. In total, three washing steps were performed. Finally, the reporter activity was measured in a Berthold chain luminometer. The luciferase activity was measured with 300 µl luciferase substrate for 5 second. For measurement of SEAP activity, 200 µl 1:5 diluted SEAP substrate in SEAP buffer was added to each sample, incubated for 20 minutes and measured for 10 seconds. Chemiluminescence (MACN) activity was measured by injection of 200 µl 0.06% H_2O_2 and 200 µl 0.2 M NaOH followed by 5 second measurement.

Table 2-12: Buffer composition for autoantibody assay.

buffer	**Composition**
binding buffer	50mM KH_2PO_4 / K_2HPO_4 pH7.5, 100mM NaCl, 10% Glycerol, 1% Triton X-100, 5 mg/ml BSA
Poros A	10% in PBS / 1% Triton X-100
wash buffer	50 mM KH_2PO_4/K_2HPO_4 pH 7.5, 100 mM NaCl, 0.1% Triton X-100
luciferase substrate	25 mM Glycyl-Glycin, 7.5 mM $MgSO_4$, 2 mM EGTA, 7.5 mM KPO_4, pH 7.8 with NaOH, then added to 5 mM DTT, 1 mM ATP, 0.1 mM Luciferin
20x PBS	160 g NaCl, 4 g KCl, 28.8 g Na_2HPO_4 x $2H_2O$, 4 g KH_2PO_4, dH_2O to 1 L, pH 7.5
1x PBS	1:20 dilution of 20x PBS with dH_2O
SEAP substrate	Tropix CSPD Ready to use with Sapphire II, Applied Biosystems, USA
SEAP buffer	100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM Mg^{2+}

Table 2-13: Antibodies used as positive controls for the autoantibody assays.

Antibody	Applied concentration	Supplier	Article number
anti-human BMP2 polyclonal (rabbit)	0.1 mg/ml	Dianova, Hamburg	CYT-26591
anti-human BMP7 monoclonal (mouse)	0.1 mg/ml	Immunization Unicus, Greifswald; Isolation InVivo, Hennigsdorf	631/H7, AK2341/01
anti-human BMP7 monoclonal (mouse)	0.1 mg/ml	Immunization Unicus, Greifswald; Isolation InVivo, Hennigsdorf	631/E1, AK2342/01
anti-human BMP7 monoclonal (mouse)	0.1 mg/ml	Immunization Unicus, Greifswald; Isolation InVivo, Hennigsdorf	631/G12, AK2343/01
anti-human IGF1 monoclonal (mouse)	0.1 mg/ml	Abcam, Cambridge, UK	ab40789
anti-insulin polyclonal (guinea pig)	0.1 mg/ml	Abcam, Cambridge, UK	ab7842

2.9 BMP reporter assay

To test for potential interfering or stimulating effects of positively identified autoantibodies on BMP signalling, a BMP-responsive-element (BRE) reporter plasmid (kindly provided by Prof Dr Peter ten Dijke (Korchynskyi and ten Dijke, 2002)) was used. Murine NIH3T3 cells were cultured in standard medium (DMEM/F12, 10% FBS). Cells were seeded on 96-well assay plates (white, clear bottom, Corning Incorporated, NY, USA) at 10,000 cells per well. The next day, cells were transiently transfected with the BRE reporter and pSEAP2-control plasmid (Clontech, Mountain View, USA) used for normalization purposes. Both plasmids were mixed with 40-kDa linear polyethylenimine reagent (PEI-40), incubated for 15 minutes at RT and then added to the NIH3T3 cells. 24 hours after transfection, the cell culture medium was exchanged for FBS-free medium, the IgG preparations from BMP7-aAB or BMP2-aAB positive or negative patients were added, and the cells were stimulated with rhBMP7 (0.5 nM, f.c.) or rhBMP2 (0.5 nM, f.c.). All experiments were performed in sextuplicates for BMP7 stimulation and in triplicates for BMP2 stimulation. After 24 hours of stimulation, the supernatants were collected and SEAP activity was determined using the SEAP substrate Tropix CSPD (Applied Biosystems) pre-diluted 1:5 in substrate buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM Mg^{2+}).

After 20 min of incubation, SEAP activity (relative light units, RLU) was measured for 2 sec per well in a plate luminometer. BRE reporter activity was determined after cell lysis from the homogenates by injection of 30 µl firefly luciferase substrate. Relative light units (RLU) were measured for 2 seconds, normalized to the according SEAP activity and compared to the un-stimulated control.

2.10 Serum samples

Serum samples from a cohort of 200 anonymized healthy donors (100 males and 100 females, age range; 21 to 40 years) were obtained from a commercial supplier (Invent GmbH, Biotechnology Center Hennigsdorf, Germany). Serum samples from fracture patients were collected at the Department of Orthopedics and Trauma Surgery, Heidelberg University Hospital. Two time points were analysed from 265 fracture patients (189 females, 76 males), yielding a total collection of 530 samples. The first time point was around the surgical intervention (either pre-surgery or two days after surgery), and the second time point was approximately four weeks post-surgery. The patients were categorized into different groups according to whether they have been treated by rhBMP7 or not. Additionally, information such as fracture type, previous surgeries, infections, former diseases and other anthropometric data were recorded. This study was conducted in accordance with the declaration of Helsinki. All individuals were in accordance with the study protocol and provided informed consent. The study was approved by the ethics committee of the Ruprecht-Karls-University of Heidelberg (S-636/2011).

Serum samples of diabetic patients were kindly provided by Phillip Gorden, M.D., Clinical Endocrine Branch, National Institutes of Health, Bethesda, Maryland and are described in (Malek et al., 2010).

2.11 Statistics

Whenever possible all experiments were performed in duplicate. Due to limited sample availability, for example in the analysis of human serum cohorts, only n=1 was possible. In that case a portion of samples was always measured in duplicate to control assay performance. As criterion for autoantibody positivity, a floating cut point of $P_{0.75}$+1.5*IQR was calculated for each dataset and the

median set to 1 relative unit (rel unit). The Chi-square test was used to compare two categorical variables, e.g. the incidence of aAB positivity or negativity between patients with different treatments. The result of the Chi-square test was always displayed as *p*-value and statistical significance was given and indicated as follows $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

Cell culture reporter experiments were performed in sextuplicates, tested for normal distribution by Shapiro-Wilks test and the mean reporter activities of the different conditions were compared by students´ t-test. Statistical significance was given and indicated as follows $p < 0.001$ (***).

3 Results

Autoantibodies may affect the incidence and course of human disease or may be unrelated to pathophysiological processes. The presence and relevance of aAB against growth factors and their receptors in terms of bone healing has however not been addressed before. To analyse the molecular mechanisms and clinical relevance of growth factor-relevant aAB, novel quantitative in vitro assays were developed allowing aAB to be measured in serum samples from fracture patients.

3.1 Autoantibodies against the Insulin-like growth factor 1 receptor and insulin receptor in sera of fracture patients

Autoantibodies against the Insulin-like growth factor 1 receptor (IGF1R) and insulin receptor (IR) were measured in sera of fracture patients. The assays to detect such aAB were developed prior to this study and thoroughly validated (Minich et al., 2012; Welsink Tim, 2013; Welsink, 2015). Briefly, the detection method is based on the incubation of serum samples with IGF1R-/IR-firefly luciferase fusion proteins. Bound aAB-fusion protein complexes are precipitated, with the subsequently measured luciferase activity corresponding to the amount of aAB present in the sample. The authors analysed the reproducibility and demonstrated an inter-assay coefficient of variation (cv) of 13.6% and an intra-assay cv of 9.9% for the IGF1R-aAB assay as well as an inter-assay cv of 18.4% and an intra-assay cv of 9.1% for the IR-aAB assay (Welsink Tim, 2013). The signal is linear over a serial dilution (measured up to one magnitude). No matrix effect was observed, this being determined by mixing aAB positive and negative sera (Minich et al., 2012). The colleagues found IGF1R- and IR-aAB in approximately 5-10 % of patients and controls when using a cut-off criterion of mean + 3SD.

Based on the above background two working hypotheses were generated. First, the prevalence of IGF1R- and IR-aAB is higher in fracture patients that show bone regeneration complications. Second, the 5% of the population that carry IGF1R- or IR-aAB are at greater risk for a compromised bone healing situation.

The results of the IGF1R- and IR-aAB measurement in sera of fracture patients are shown in Figure 3-1. Using the cut off criterion of mean plus three standard deviations excluding the 10% highest values (mean + 3SD, blue line), previously defined by Welsink et al., 36 samples (7%) were detected aAB

positive for both the IGF1R- and the IR-aAB assay. Of these, 14 samples were double positive for aAB against both receptors, i.e. nearly half of the positive samples.

Here, a new criterion is introduced as a cut point for autoantibody positivity, the 75th percentile ($P_{0.75}$) plus 1.5 times the interquartile range (IQR). This calculation includes all data points measured in the data set and is a criterion used in statistics to exclude outliers in a box plot. Using the criterion of $P_{0.75}$+1.5*IQR for aAB positivity (red line), 25 samples (5%) proved IGF1R-aAB positive in the IGF1R-luc aAB assay (A). In the IR-luc aAB assay, 29 samples (6%) were detected as IR-aAB positive (B). Approximately half of the positive samples (11 samples) were double positive for both IGF1R- and IR-aAB. The actual counts are summarized in Table 3-1. Pearson Chi-square analysis revealed a *p*-value < 0.001, meaning more samples were double positive than would be expected on a statistical basis. With these group sizes the statistically expected value would be 1, instead of the 11 double positive samples observed.

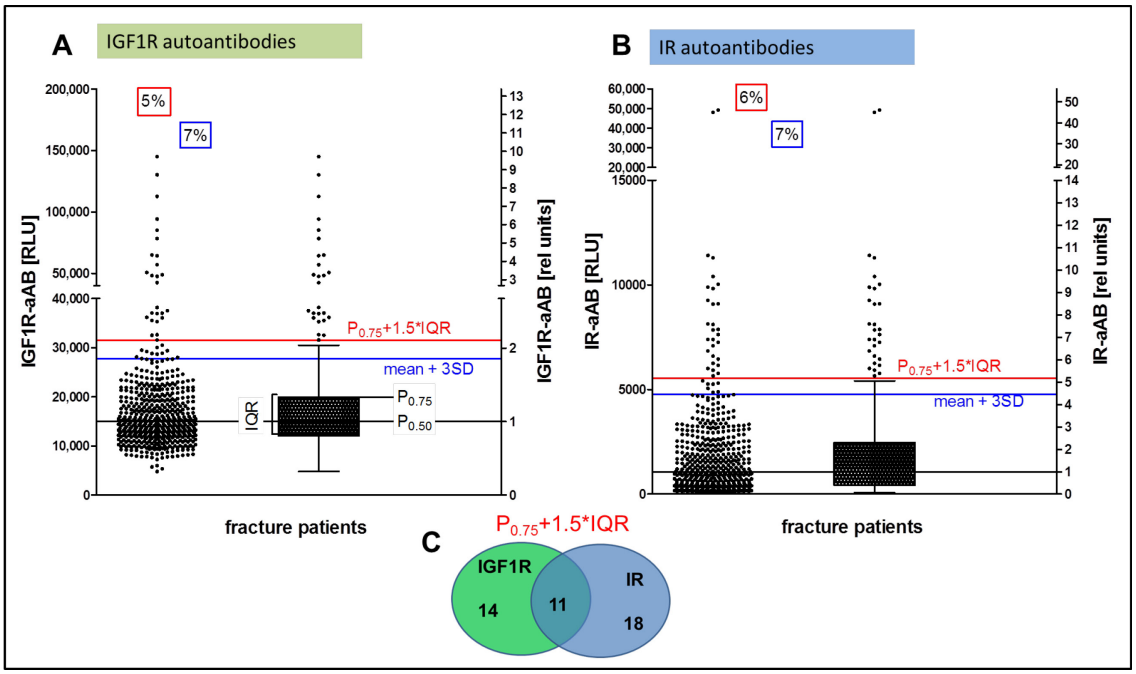

Figure 3-1: IGF1R- and IR-aAB in fracture patients. (A) IGF1R-aAB in serum samples from fracture patients were measured by IGF1R-luciferase aAB assay (n=530). Positivity was given for 36 samples (7%) using the cut point mean + 3SD (blue line) and for 25 samples (5%) exceeding the cut point $P_{0.75}$+1.5*IQR (red line), respectively. (B) Analysing the same samples by the IR-luciferase aAB assay, again 36 samples (7%) were detected positive using mean + 3SD and 29 samples (6%) using $P_{0.75}$+1.5*IQR (n=529). (C) About half of the positive samples (11 samples) were double positive for both IGF1R- and IR-aAB. The assay output is relative light units (RLU, left y-axis). The values were divided by the median resulting in aAB relative units (rel units, right y-axis). Red line: assay cut point 75th percentile ($P_{0.75}$) plus 1.5 times the interquartile range (IQR). Blue line: assay cut point mean plus three standard deviations excluding the 10% highest values. Black line: median.

Table 3-1: Cross-tabulation of double negative, IGF1R-aAB positive, IR-aAB positive and IR-/IGF1R-aAB double positive samples using the cut-off criterion $P_{0.75}$+1.5*IQR for aAB positivity.

		IGF1R-aAB [n]		Total
		neg	pos	
IR-aAB [n]	neg	486	14	500
	pos	18	11	29
Total		504	25	529
Pearson Chi-Square		$p < 0.001$		

IGF1R- and IR-aAB were analysed at two different time points after surgery (Figure 3-2). At the time of surgery, 4% of the patients were positive for IGF1R-aAB, rising to 6% four weeks later (A). For IR-aAB 5% of the patients were positive at surgery and 6% four weeks later (B). The dotted lines indicate that in most cases the same patients stayed positive with only little variation of their aAB titres. There was no obvious influence of the intervention on the prevalence of IGF1R- and IR-aAB. When comparing the prevalences of aAB-positive patients between the two time points by Chi square analysis, no difference in aAB occurrence was statistically determined reflected by p = 0.421 for IGF1R-aAB and p = 0.334 for IR-aAB, respectively (Table 3-2).

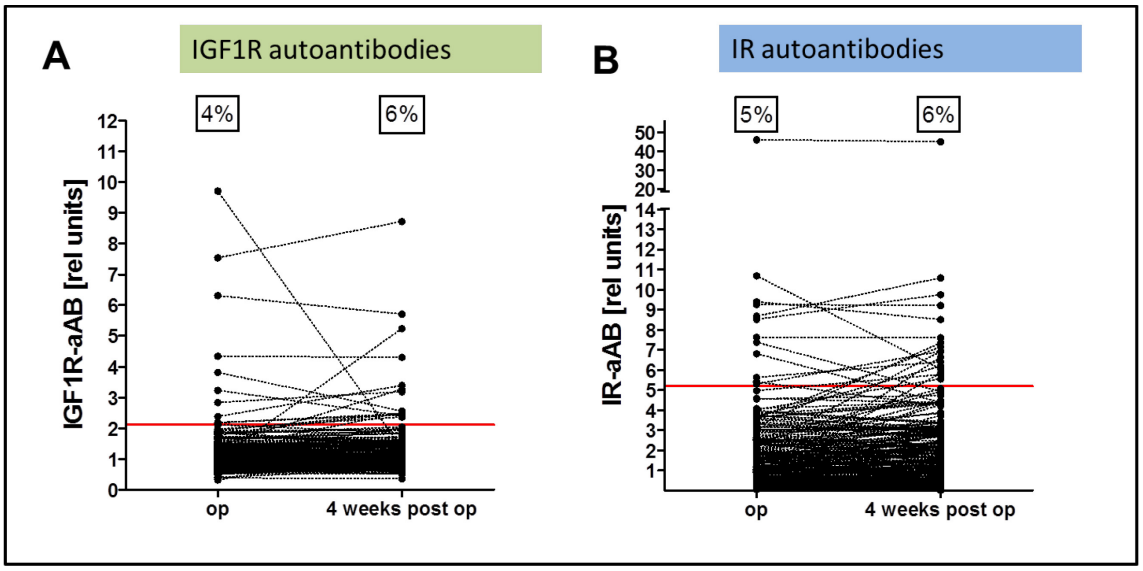

Figure 3-2: IGF1R- and IR-aAB detection in fracture patients at different time points. (A) 4% of the patients are IGF1R-aAB positive at surgery (op) and 6% four weeks later. The development over time of each individual is indicted by the dashed lines. (B) IR-aAB measurement shows 5%

of the patients being IR-aAB positive at surgery and 6% of the patients four weeks later exceeding the cut point of $P_{0.75}$ + 1.5* IQR.

Table 3-2: Statistical analysis of IGF1R- and IR-aAB occurrence between different time points of blood withdrawal from fracture patients.

	IGF1R-aAB		**IR-aAB**	
	op	**4 week**	**op**	**4 week**
neg [n]	254	250	253	247
pos [n]	11	15	12	17
total [n]	265	265	265	264
Pearson Chi square	p = 0.421		p = 0.334	

To analyse the course of IGF1R- and IR-aAB titres over time, more consecutive serum samples of selected individuals were available (Figure 3-3). IGF1R-aAB were measured in two to five time points of ten patients, who were previously identified as being IGF1R-aAB positive and in four of the IGF1R-aAB negative fracture patients. (A) Intra-individual measurements showed only a few fluctuations and the titres remained relatively stable over a time course of up to half a year. Whereas, the inter-individual differences of IGF1R-aAB titres in the positive patients were considerably higher. The red line demonstrates the $P_{0.75}$+1.5*IQR of the negative samples. One patient (# 9, brown line) does not exceed this threshold for all time points measured. Also, patients # 5 (blue line) shows values higher than the threshold only for the first serum sample and not for the next time points (A). In IGF1R-aAB negative patients the signal stayed on background level over time (C). IR-aAB were measured in eight IR-aAB positive and four IR-aAB negative patients. In general, IR-aAB positive patients stayed positive over the time course. Patient # 22 was measured as IR-aAB negative at all time points and patients # 18 was identified above the $P_{0.75}$+1.5*IQR of the negative samples at the first time point measured and dropped then underneath the threshold in all the follow-up serum samples (B). In IR-aAB negative patients signals stay under the cut point for all time points measured (D).

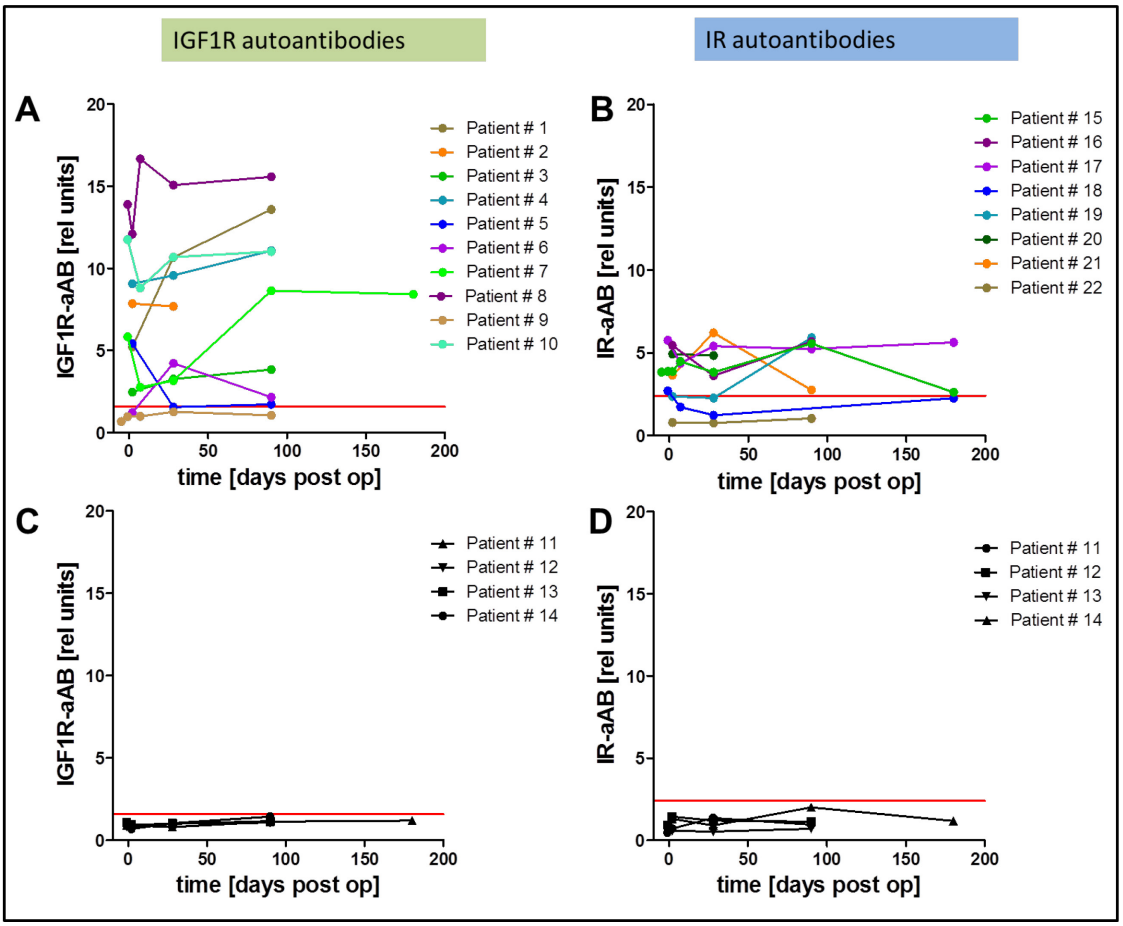

Figure 3-3: IGF1R- and IR-aAB trend in fracture patients over time. (A) IGF1R-aAB positive patients show different titres between individuals but only little differences in individual titres over time. (B) IR-aAB show lower titres over time and most of the patients stay aAB positive in the time course. (C) IGF1R-aAB negative patients maintain signals at background level. (D) IR-aAB negative patients again show slightly more variation in background signals indicating a higher variability of the aAB or this assay, albeit in the noise range only. The red line indicates the $P_{0.75}$+1.5*IQR of the negative samples.

Last, the clinical relevance of IGF1R- and IR-aAB was addressed by comparing the occurrence of aAB in correlation to the healing outcome of the fracture patients (Figure 3-4). IGF1R-aAB were found with an incidence of 5% in both groups, those that had consolidation and those that had no consolidation (A). IR-aAB were found in 6% of the patients that healed completely and in 4% that had disturbed healing (B). Based on these findings, there was no direct relationship between IGF1R- and IR-aAB and the healing outcome.

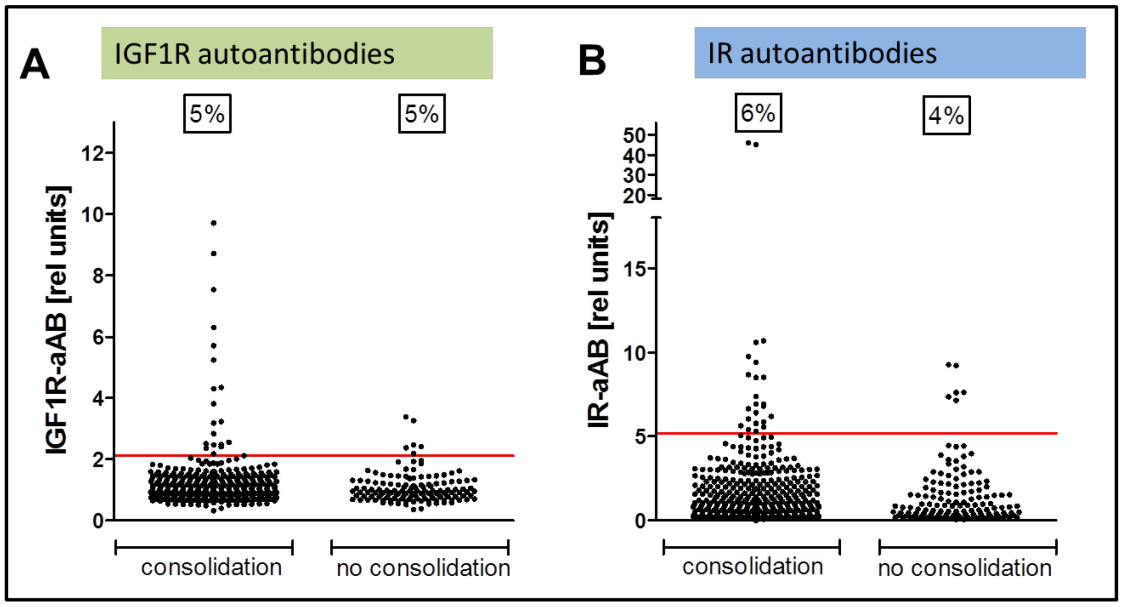

Figure 3-4: IGF1R- and IR-aAB in fracture patients according to their healing outcome. (A) The prevalence of IGF1R-aAB is the same (5%) for patients with and without consolidation of their fractures. (B) IR-aAB were found in 6% of the patients with successful healing and 4% in the unhealed group.

In summary, IGF1R-and IR-aAB were found in sera of fracture patients with a similar prevalence as in other patient cohorts and healthy controls. The titres were stable over time. A direct correlation between aAB presence and fracture healing outcome was not observed.

3.2 Development of BMP7 and BMP2 autoantibody detection assays

Bone morphogenetic proteins are important growth factors involved in bone healing. Impaired bone healing may occur through insufficient activity of BMP. Autoantibodies against these growth factors could be a reason for impaired healing capacity.

In order to measure aAB against BMP in human serum, it was necessary to establish a detection assay for BMP-aAB since such an assay does not exist on the market. The assay principle used in this work is based on the binding of the aAB in human serum to MACN-labelled recombinant human BMP (rhBMP), shown for BMP7-MACN in Figure 3-5. The immunoglobulins, including the aAB bound to BMP7-MACN, are precipitated by Protein-A-containing Poros A. After several washing and centrifugation steps the chemiluminescence of the bound MACN is measured and corresponds to the presence and titre of BMP7-aAB in the serum.

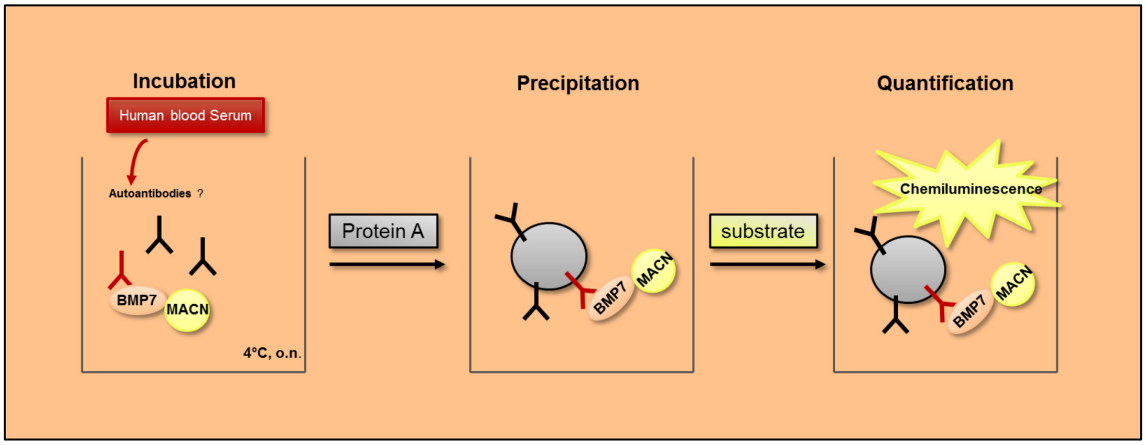

Figure 3-5: BMP aAB assay. A dilution of MACN-labelled BMP (shown for BMP7-MACN) is incubated with a human serum sample potentially containing aAB against BMP. BMP-aAB bind BMP-MACN. By adding a Protein A slurry immunoglobulins are precipitated via binding of their Fc-part including the BMP-MACN-bound aAB. After three washing and centrifugation steps the presence of BMP-aAB is detected by a chemiluminescence reaction of the bound MACN label.

Each step of the assay was optimised during development. BMP7 and BMP2 were used directly from the manufacturers as it is used for treatment in the clinics. After labelling of the proteins with MACN as bait for the detection of aAB their chemiluminescence was measured and compared to the unlabelled (cold) BMPs (Figure 3-6). Both BMP7-MACN and BMP2-MACN preparations show high chemiluminescence activity in the range of several million RLU per µl (A). When performing the BMP2-aAB assay under optimized conditions with a dilution series of a commercial available BMP2-aAB a nearly linear decrease of the measured RLU signal is observed with decreasing concentration of 1-100 µg/ml anti-BMP2-antibodies in solution (B). A similar result was determined by performing the BMP7-aAB assay with a dilution series of anti-BMP7-antibody (C). Applying the BMP7-aAB assay for three BMP7-aAB positive and three negative human sera, a dynamic range in RLU signal activity is observed up to a dilution factor of 1:8 (D). When diluting BMP7-aAB negative sera with PBS a steady signal at background level is detected. These experiments show the high dynamic range of the two assays being able to discriminate between aAB positive and negative samples and to quantify aAB titres by signal strength. The small error bars underline the reproducibility by low intra-assay variation.

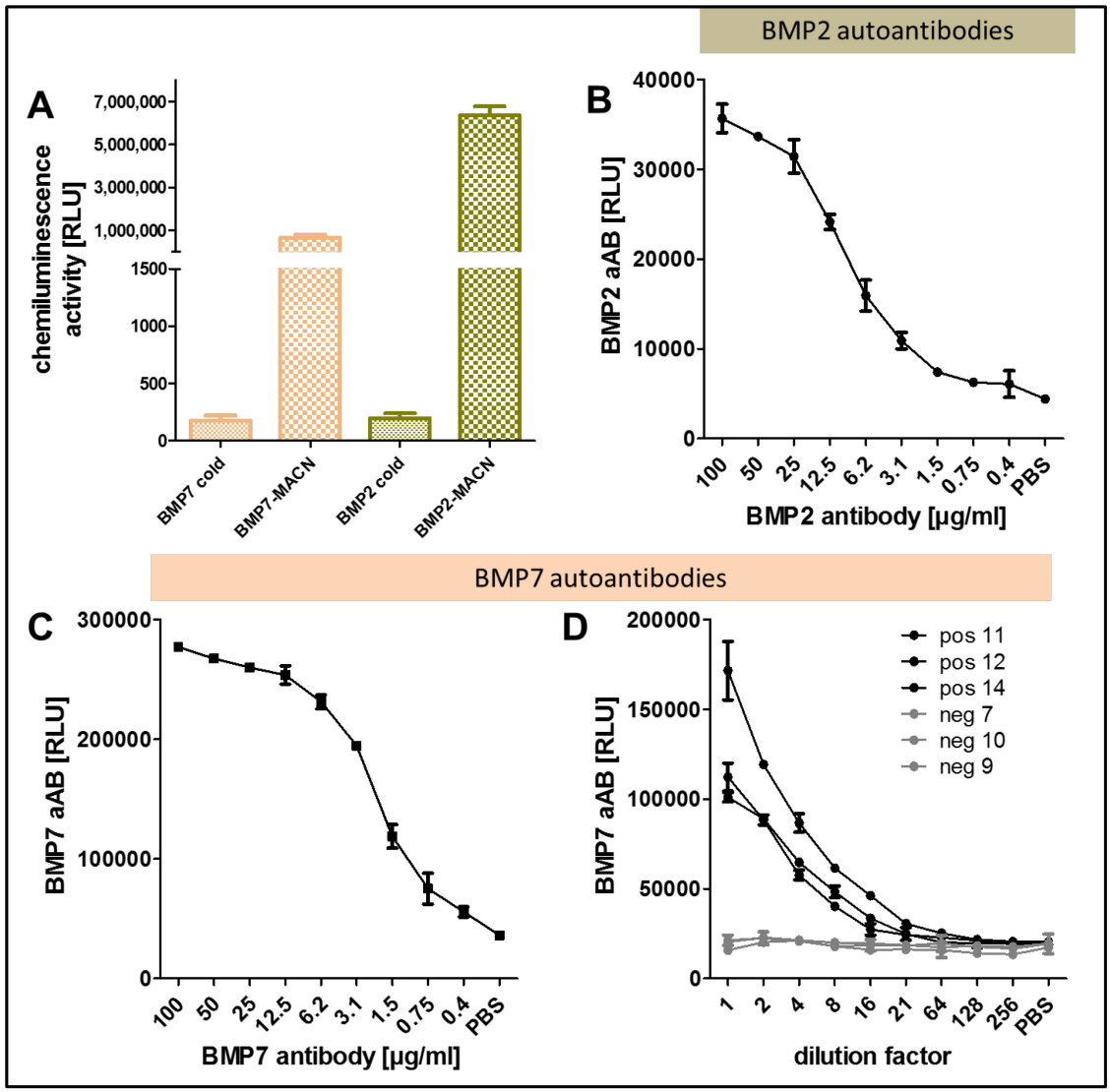

Figure 3-6: BMP autoantibody assay characterization. (A) After labelling BMP7 and BMP2 with MACN the protein solutions show high chemiluminescence activity upon reaction with the MACN substrate H_2O_2 as compared to preparations of unlabelled BMP7 and BMP2. These preparations are applied in the aAB detection assays as bait for the aAB. (B) Using a serial dilution of a commercially available BMP2-antibody the BMP2-aAB assay shows a dynamic range of signal intensity of over one order of magnitude. (C) Applying a monoclonal BMP7-antibody to the BMP7-aAB assay a similar nearly linear decline of signal intensity can be observed with declining BMP7-ab concentration. (D) Serial dilutions of three BMP7-aAB positive human serum samples show a linear range up to a PBS dilution of 1:8 in the BMP7-aAB assay. Whereas serial dilutions of BMP7-aAB negative serum samples show stable RLU signals at background level. n=2, mean +/- SD (Schuette et al., 2016).

In order to evaluate whether BMP-aAB are present in the normal population and to determine their prevalence, serum samples from 200 healthy donors were purchased from a commercial supplier. The demographics of the probands are summarized in Table 3-3. The cohort consists of 100 females and 100 males in

the age range of 21 to 40 years so that samples were matched for gender and age.

Table 3-3: Demographics of control sera of 200 healthy probands purchased from a commercial provider.

Invent sera	**female**	**male**
patients [n]	100	100
age range [years]	21-40	21-40
median age [years]	30.5	30.5

The prevalence of BMP-aAB in healthy probands is shown in Figure 3-7. The cut point for aAB positivity was determined by calculating the 75th percentile ($P_{0.75}$) of all values plus 1.5 times the interquartile range (IQR). BMP7-aAB measurement identified five samples being above the cut point and were therefore considered BMP7-aAB positive (A). Five positive samples equal a prevalence of 2.5% for the whole study population. BMP2-aAB measurement in the same samples revealed five samples (2.5%) being BMP2-aAB positive (B). However, these samples exceeded the cut point only slightly whereas BMP7-aAB positive samples showed a positivity of up to four times the median (black line). One of the positive samples is double positive for both BMP7- and BMP2-aAB. The sample numbers are too small to perform Chi square analysis, which requires a minimum of five samples per group.

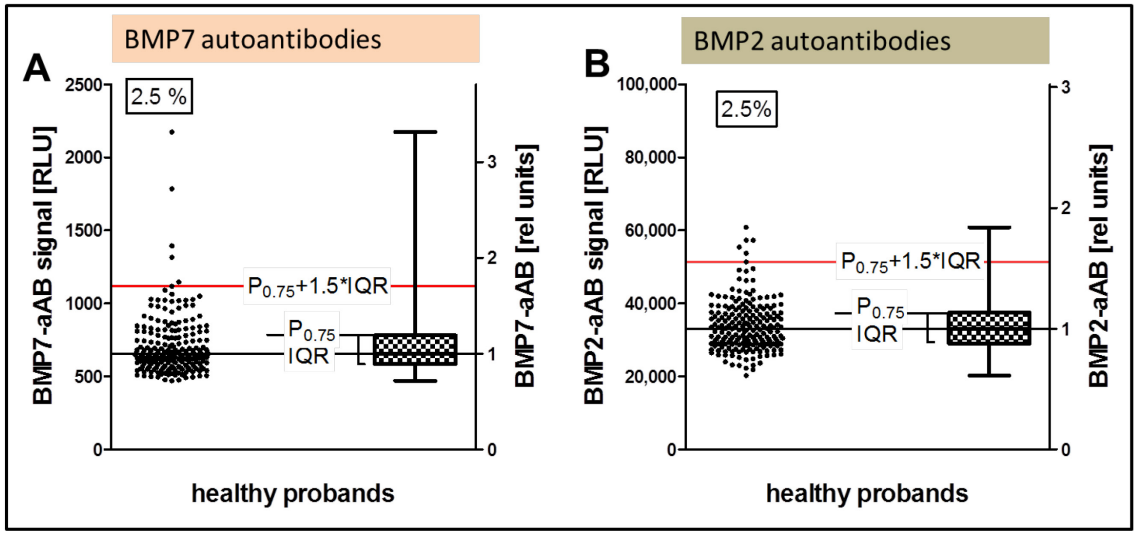

Figure 3-7: BMP7- and BMP2-aAB prevalence in the healthy population. Serum samples from 200 healthy volunteers were measured for BMP7-aAB (A) and BMP2-aAB (B). Each dot

represents one measurement of one subject. For both assays five samples (2.5%) exceeded the cut point of the 75th percentile plus 1.5 times the interquartile range ($P_{0.75}$+1.5*IQR) (red line). The median is indicated by the black line (Schuette et al., 2016).

3.3 BMP7 autoantibodies are induced in fracture patients treated with rhBMP7

BMP-autoantibodies were measured in serum samples of fracture patients. A cohort of 265 patients was available. The patients had different fracture healing outcomes and various previous fracture surgeries. Fresh fractures without prior treatment served as controls. In this study, the patients were divided into two groups, either rhBMP7 treated or not, summarized in Table 3-4. These groups consisted of different subgroups that received different combinations of therapeutic options to induce fracture healing.

Table 3-4: Different treatment options applied to fracture patients.

BMP7 treatment	Patients [n]	Treatment groups	Patients [n]	Consolidation/ no consolidation [n]	BMP7-aAB over time, patients [n]
Fracture treatment w/o BMP7	178	Fresh fractures	145	104/41	5
		Pseudarthrosis	33	32/1	1
Fracture treatment with BMP7	87	Pseudarthrosis	38	26/12	2
		Pseudarthrosis RIA [a] or Spongiosa	21	17/4	2
		Pseudarthrosis Masquelet technique [b]	28	18/10	3

[a] RIA: reamer irrigator aspirator.
[b] Masquelet: use of temporary cement spacer.

BMP-aAB results in the sera of fracture patients are depicted in Figure 3-8. Using the BMP7-aAB detection assay 26 samples exceeded the cut point (red line) and were considered BMP7-aAB positive (A). This amount equals 5% of all samples. Negative samples show a steady signal at background level. Analysing the same samples in the BMP2-aAB assay 14 samples had values above the cut point which is a prevalence of 2.5% (B). The results of the BMP-

aAB measurements are summarized in Table 3-5. Four samples were double positive for BMP7- and BMP2-aAB. Pearson Chi square analysis gives a *p*-value < 0.001, indicating a highly significant interaction between the aAB in positive patients. The statistically expected count for this group size is one double positive sample. This points towards a connection between BMP7- and BMP2-aAB positivity.

Compared to the prevalence of BMP-aAB positivity in the normal population (chapter 3.2) BMP2-aAB in sera of fracture patients show the same prevalence as the healthy population (2.5%) whereas BMP7-aAB show a higher prevalence of 5% aAB positives in fracture patients and 2.5% positives in healthy probands.

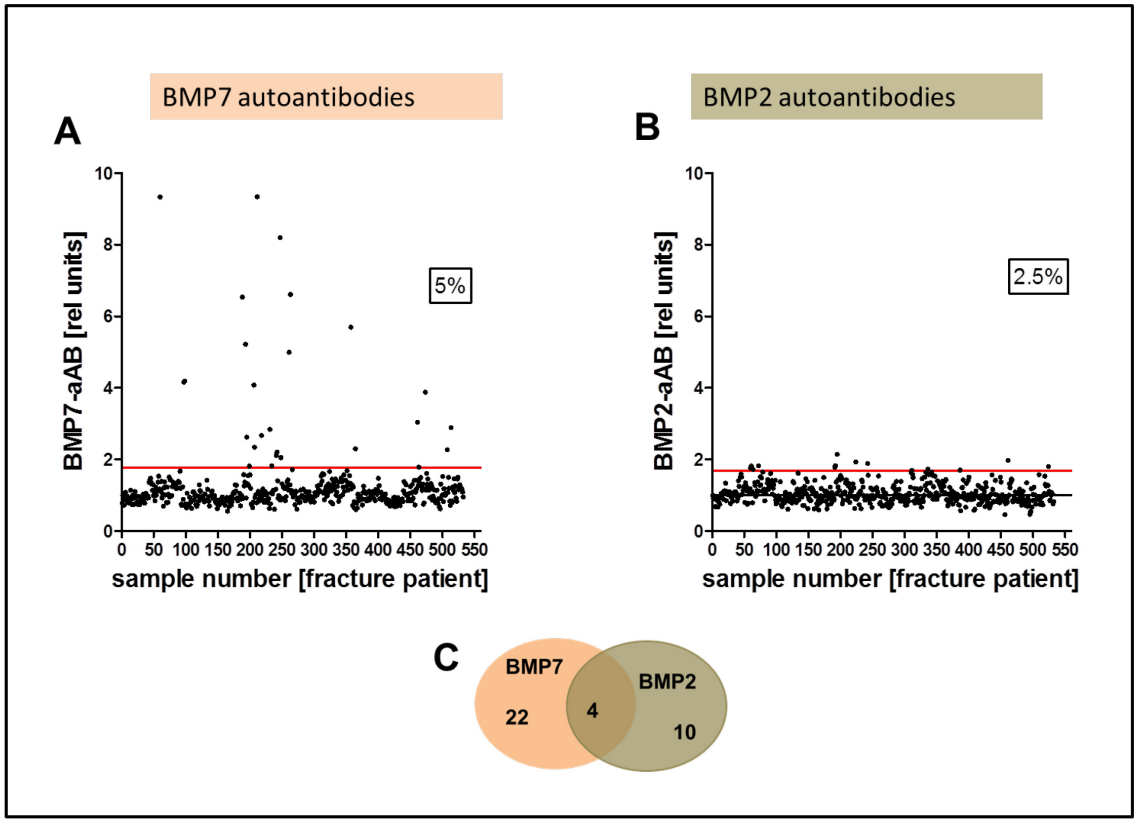

Figure 3-8: BMP-aAB measurement in fracture patients. (A) In 530 samples from fracture patients BMP7-aAB were measured. Of these, 26 samples (5%) exceeded the cut point of $P_{0.75}$+1.5*IQR (red line) and were considered BMP7-aAB positive. Some samples showed signals up to 10 times above the median (black line). (B) BMP2-aAB measurement in the same samples showed 14 (2.5%) samples to be BMP2-aAB positive. These samples however exceeded the cut point only slightly. (C) Four of the positive samples were positive for both BMP7- and BMP2-aAB.

Table 3-5: Summary of BMP7- and BMP2-aAB positive samples and double positive samples of fracture patients.

		BMP7-aAB [n]		Total
		neg	pos	
BMP2-aAB [n]	neg	494	22	516
	pos	10	4	14
Total		504	26	530
Pearson Chi square		$p < 0.001$		

In order to determine the induction of BMP-aAB by fracture treatment, the patients were divided into two groups: either rhBMP7 treated or not. Two different time points were analysed, at surgery (op) and four weeks after surgery (4 w post op) (Figure 3-9). BMP7-aAB measurement shows 1% of the patients being positive for BMP7-aAB in the rhBMP7-naïve group at both time points (A). The development of each individual is indicated by the dotted lines. In the rhBMP7-treated group, 6% of the patients were BMP7-aAB positive at surgery. Four weeks later the number increased to 18% BMP7-aAB positive patients. The aAB titres also increased demonstrated by higher relative units. BMP2-aAB measurement detected 2% BMP2-aAB positive patients in the rhBMP7-naïve group at both time points and in the treated group at surgery with low titres exceeding the cut point only slightly (B). Nevertheless, four weeks after surgery in the treated group an increase of BMP2-aAB positive samples to 6% is observed. The treatment with rhBMP7 therefore seems to increase the number of BMP2-aAB positive samples. However, only low titres were detected. The patient numbers of each group and the amounts of BMP-aAB positive samples are listed in Table 3-6 and Table 3-7. Comparison of the groups by Chi square test showed a statistical significance of aAB positivity between the time points op and four weeks after op just for BMP7-aAB in the rhBMP7-treated group with $p = 0.010$.

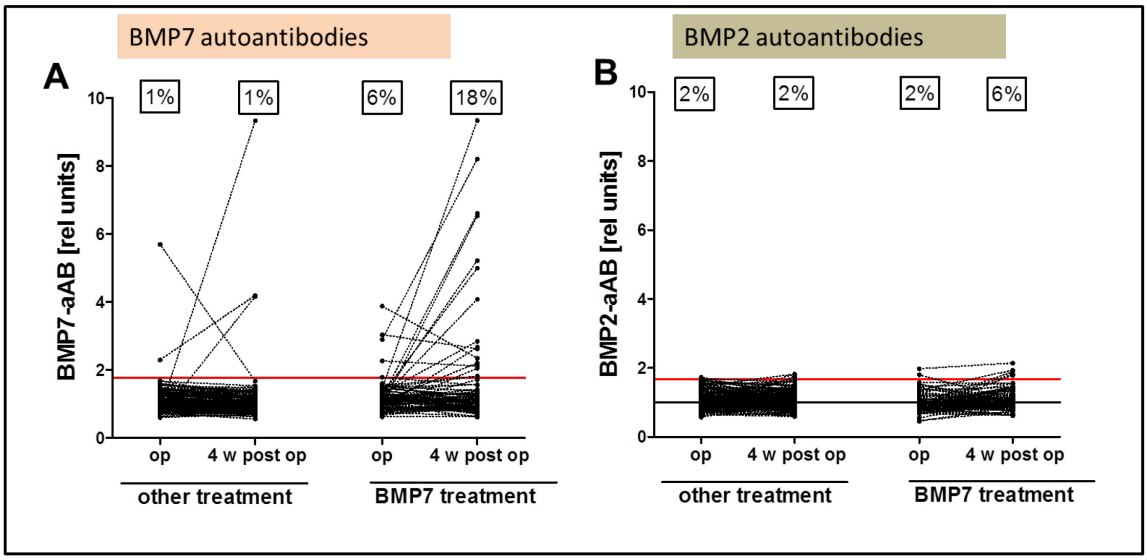

Figure 3-9: BMP-aAB development in fracture patients treated or not with rhBMP7. (A) The rhBMP7-treated group has a higher incidence of BMP7-aAB positive patients with 6% at surgery (op) and 18% four weeks later compared to 1% for the BMP7-naïve group at both time points. (B) The BMP2-aAB measurement revealed only very low signals with 2% BMP2-aAB positive samples in the BMP7-naïve group at both time points and also at surgery in the BMP7-treated group. Four weeks later again an increase in BMP2-aAB positive patients to 6% is determined (Schuette et al., 2016).

Table 3-6: Chi square analysis of BMP7-aAB in different treatment groups.

	BMP7-aAB			
	other treatment		**BMP7 treatment**	
	op	**4 week**	**op**	**4 week**
neg [n]	176	175	82	71
pos [n]	2	3	5	16
total [n]	178	178	87	87
pos [percent]	1%	1%	6%	18%
Pearson Chi square	$p = 0.652$		$p = 0.010$	

Table 3-7: Chi square analysis of BMP2-aAB in different treatment groups.

	BMP2-aAB			
	other treatment		**BMP7 treatment**	
	op	**4 week**	**op**	**4 week**
neg [n]	175	174	85	82
pos [n]	3	4	2	5
total [n]	178	178	87	87
pos [percent]	2%	2%	2%	6%
Pearson Chi square	$p = 0.703$		$p = 0.247$	

The course of BMP7-aAB positivity over time was analysed in patients selected from the fracture patient cohort. Serum samples of seven BMP7-aAB positive and six BMP7-aAB negative patients with three to nine time points spanning a period of up to one year follow up were measured (Figure 3-10). Mostly the BMP7-aAB titres are induced shortly after surgery. One patient (patient # 5) showed only weak positivity. In all but one case the BMP7-aAB titres dropped again after 2-3 months. In one patient (patient # 6, green) this was not observed, however only three time points up to a follow-up of three months were available of this patient. Consecutive time points of BMP7-aAB negative patients show steady signals at background level. The titres of some of the BMP7-aAB positive patients drop to the same level as the negative controls during the period measured (patient # 1, # 2, # 3 and # 7).

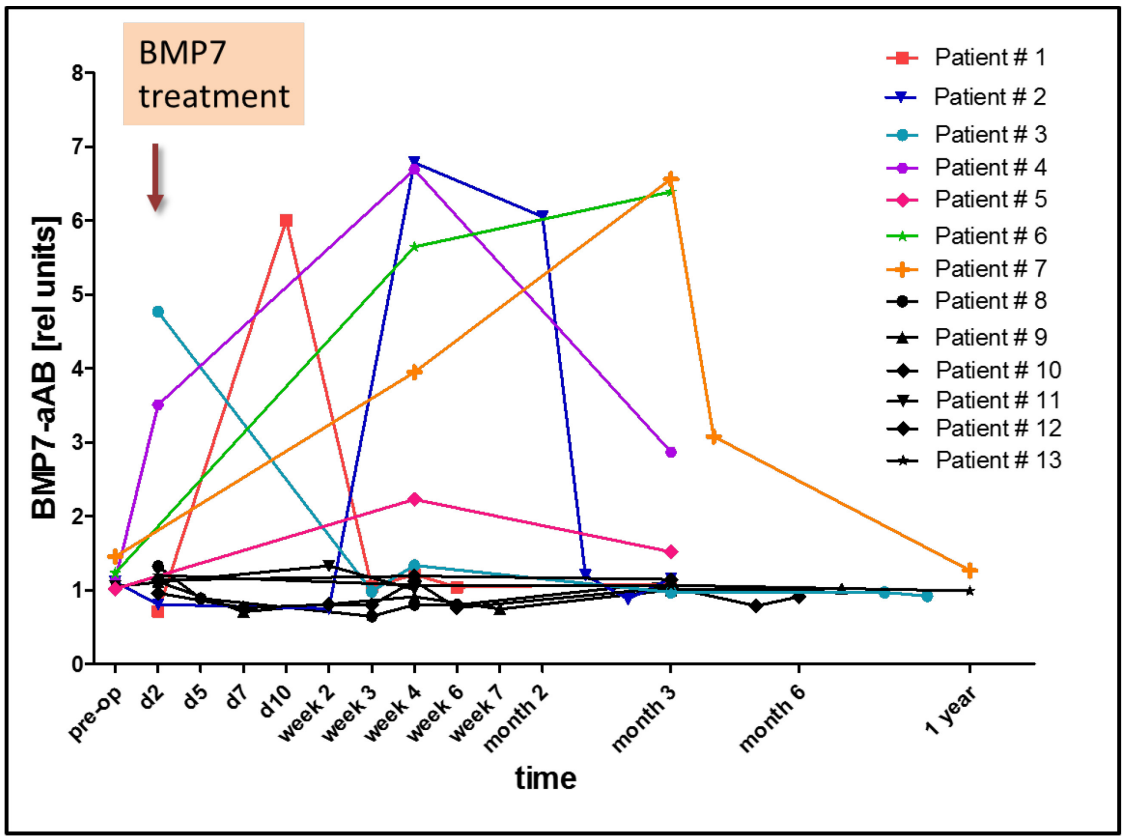

Figure 3-10: BMP7-aAB trend over time**.** The patients that were tested positive for BMP7-aAB showed a transient occurrence of aAB. In most cases BMP7-aAB positivity returned to undetectable levels within two months. In one patient a decline was not observed in the samples available for analysis (green). The negative control sera from the same study showed a steady signal at background level (black). BMP7-aAB positive sera (# 1-7), BMP7-aAB negative sera (# 8-13) (Schuette et al., 2016).

The healing outcome of the fracture patients was monitored and evaluated in the clinics. Evaluated by X-ray, non-consolidation was determined by the surgeons when the fracture gap was not closed after six months. BMP7-aAB measurement according to the healing outcome of the fracture patients is demonstrated in Figure 3-11. In the group that showed consolidation, 3% were BMP7-aAB positive at surgery and 6% four weeks later. In the group that showed no consolidation, again 3% were BMP7-aAB positive at surgery but in this case, 10% were positive four weeks later. However, the highest titres are observed in the group with successful healing. Most of the BMP7-aAB positive patients belong to the BMP7-treated group. The corresponding numbers are summarized in Table 3-8.

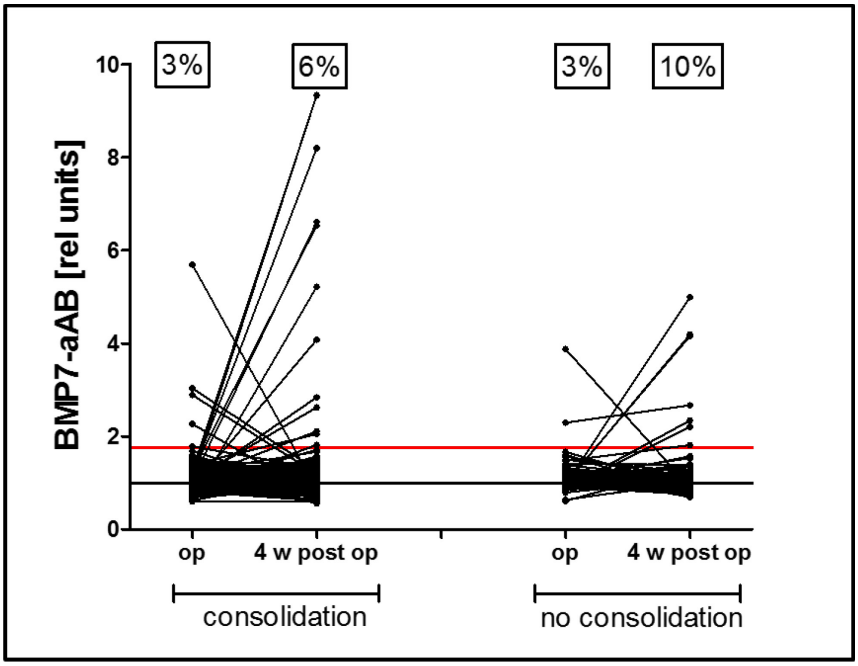

Figure 3-11: BMP7-aAB according to fracture healing outcome. Fracture patients were divided into those that had a positive healing outcome (consolidation) and those that had a negative healing outcome (no consolidation). It is observed that at surgery both groups have a BMP7-aAB prevalence of 3%. Four weeks after surgery the incidence of BMP7-aAB increased up to 6% in patients whose fractures healed later on and up to 10% in those patients whose fractures did not heal (Schuette et al., 2016).

Table 3-8: BMP7-aAB determination according to healing outcome and divided into time points.

	consolidation		**no consolidation**	
	op	**4 week**	**op**	**4 week**
BMP7-aAB neg [n]	192	185	65	60
BMP7-aAB pos [n]	5	12	2	7
total [n]	197	197	67	67
pos [percent]	3%	6%	3%	10%

To summarize the results of this chapter, Figure 3-12 shows a scheme of the findings of BMP-aAB in fracture patients. The prevalence of BMP7-and BMP2-aAB in healthy individuals and patients without rhBMP7 treatment is 1-2%. After treatment with rhBMP7 aAB levels rise up to 18% for BMP7-aAB and up to 6% for BMP2-aAB. These therapy-induced aAB are transient and disappear over time.

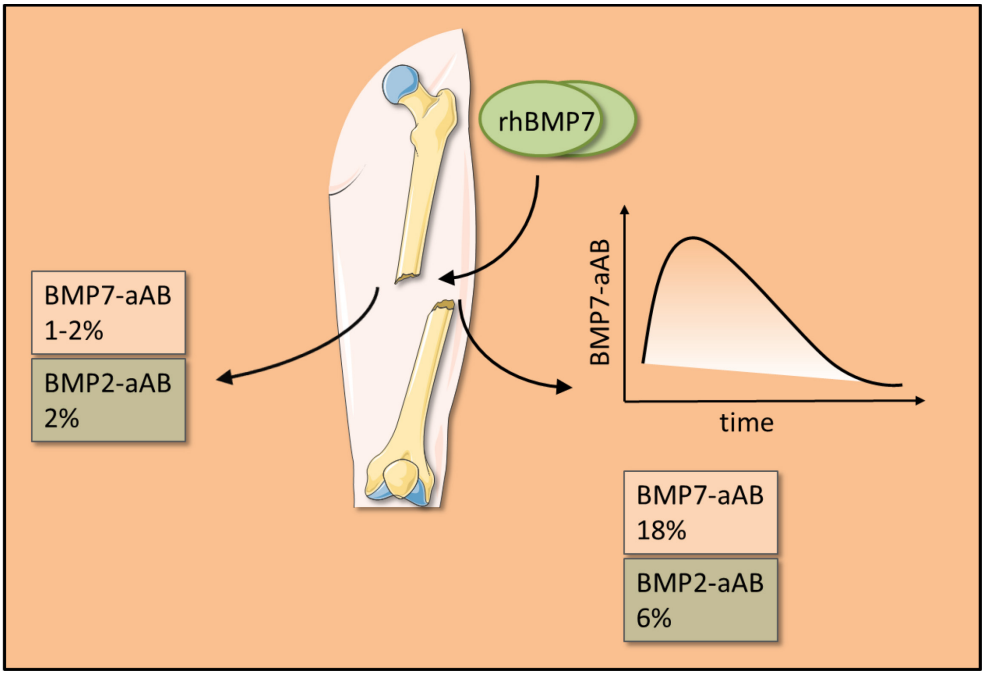

Figure 3-12: Summary of the findings by BMP-aAB measurement in fracture patients. The incidence of both BMP7-and BMP2-aAB in the normal population and without rhBMP7 treatment is about 2%. Treatment with rhBMP7 increases the occurrence of BMP7-aAB to 18% and of BMP2-aAB to 6%. These therapy-induced aAB disappear over time.

3.4 Biological effect of BMP-aAB on BMP signal transduction

The function of BMP-aAB and their molecular effect on the BMP signalling pathway was approached by a BMP reporter system in cell culture. Immunoglobulins of BMP-aAB positive and negative sera were isolated by Protein A precipitation. The obtained IgG concentrations by this method are summarized in Table 3-9 and compared to other IgG concentrations in literature determined by a commercial nephelometry assay (Gonzalez-Quintela et al., 2008). In comparison to the published range of immunoglobulin concentrations of 407–2170 mg/dl, the isolation method used here leads to a similar range of 493–1710 mg/dl, although different methods usually result in different yields. In summary, it can be assumed that the majority of IgG were isolated by our method.

Table 3-9: Reference IgG concentrations in human serum and experimental values in isolated IgG preparations.

	isolated IgG	**serum IgG (Gonzalez-Quintela et al., 2008)**
mean	848 mg/dl	1118 mg/dl
SD	348 mg/dl	251 mg/dl
median	763 mg/dl	1090 mg/dl
range	493–1710 mg/dl	407–2170 mg/dl

To confirm that the results of the BMP-aAB measurement are actually based on antibodies the BMP-aAB assay was repeated with the isolated IgG preparations (Figure 3-13). PBS served as control for the background noise. IgG 1-10 are isolated IgG from BMP-aAB negative sera. In detail, IgG 1-4 are controls from other cohorts and IgG 5-10 are aAB negative controls from the fracture patient cohort. IgG 11-20 are isolated from BMP7-aAB positive samples and IgG 19-27 are isolated from BMP2-aAB positive samples. The cut point (red line) was determined $P_{0.75}$+1.5*IQR. In the BMP7-aAB assay (A) IgG 11-14 and 18 exceeded the cut point, those samples that also showed the highest values when measuring in serum directly. The other previously BMP7- or BMP2-aAB positive samples could not clearly be distinguished from aAB-negative samples. In the BMP2-aAB assay (B) only IgG 15 and 18 showed slightly elevated values.

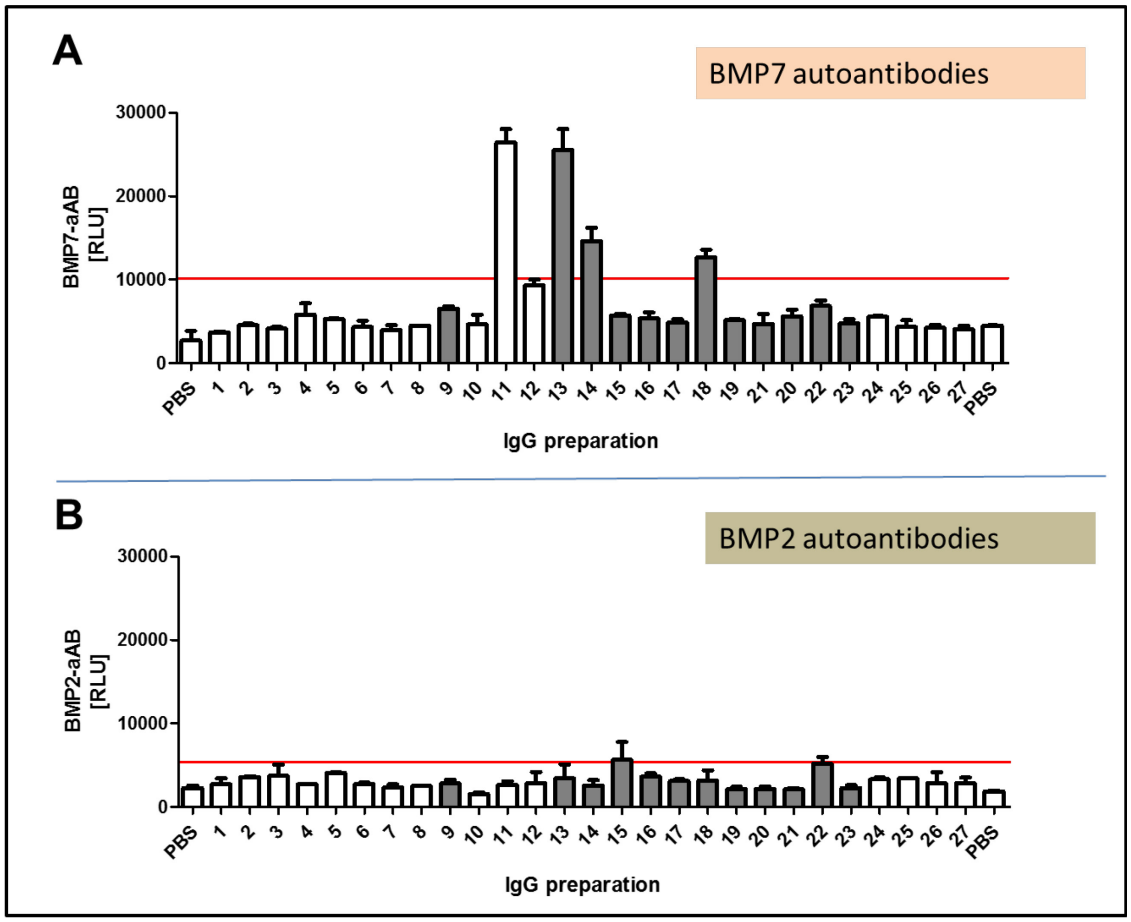

Figure 3-13: BMP-aAB measurement in isolated IgG preparations. (A) Autoantibodies were measured in isolated IgG from aAB negative controls (1-10) and from BMP7- or BMP2-aAB positive samples (11-27). The BMP7-aAB assay detected IgGs 11-14 and 18 with highest RLU values, in agreement with the results from serum. (B) The BMP2-aAB assay did not detect any clearly elevated signals. IgG 15 and 22 were slightly increased. Cut point is $P_{0.75}$+1.5*IQR (red line). Grey bars are isolated IgGs from rhBMP7-treated patients. n = 2, mean + SD.

In a next step, a BMP reporter system in cell culture was established in order to determine whether the aAB have an activating, a blocking, or a neutral effect on BMP signal propagation. A BMP-responsive plasmid (BRE) (Korchynskyi and ten Dijke, 2002) was transfected into cells together with a secreted alkaline phosphatase plasmid (pSEAP) for normalization purposes (schematic illustration Figure 3-14 A). The BMP stimulation was established by a concentration series of rhBMP7 (B) and rhBMP2 (C), respectively. The same recombinant proteins were used that are also applied in clinical treatment and that were used for the establishment of the aAB detection assays. The BRE reporter reacts to increasing concentrations of rhBMP by expressing enhanced amounts of firefly luciferase in a sigmoidal fashion. For further experiments, a concentration of 0.5 nM for both rhBMP7 and rhBMP2 were chosen as fixed

dosage (red box) since this concentration was relatively central within the dynamic range, thereby allowing the identification of both stimulating and blocking aAB. An anti-BMP7-ab or an anti-BMP2-ab was used as positive control to mimic BMP-aAB. The BRE reporter was co-stimulated with 0.5 nM rhBMP7 and different concentrations of BMP7-ab (D). The BMP7-ab has a blocking effect on the BMP signal transduction. The reduction of luciferase signal is proportional to the BMP-ab concentration. A concentration of 0.5 µg/ml was chosen for further experiments. Co-stimulation of BRE-transfected cells with 0.5 nM rhBMP2 and different concentrations of anti-BMP2-ab showed a blocking effect on the luciferase signal only at the highest concentration of 50 µg/ml anti-BMP2-ab (E). This concentration was chosen for further experiments.

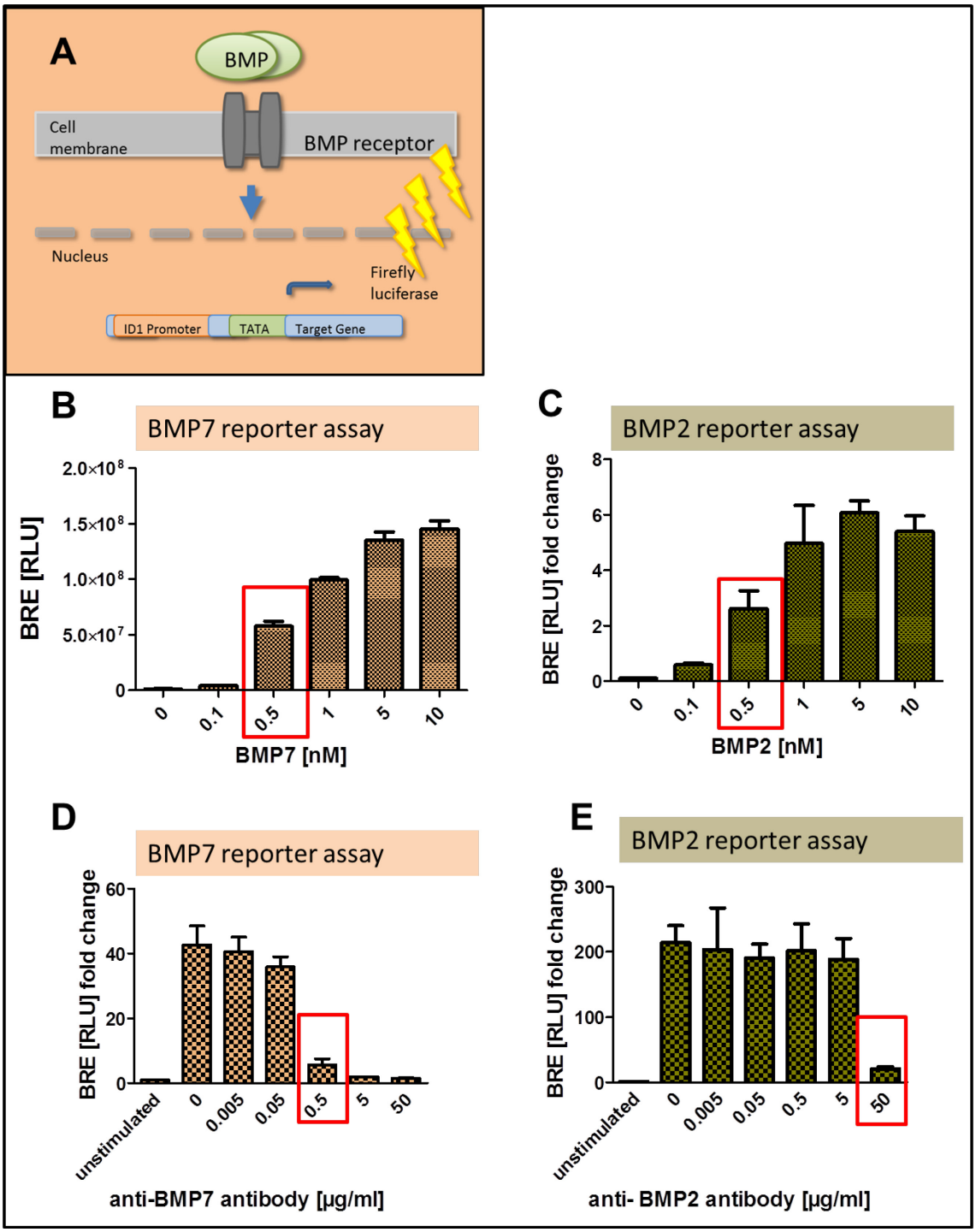

Figure 3-14: BMP7 reporter assay establishment. (A) NIH3T3 cells were transfected with a BMP-responsive Plasmid (BRE). Upon stimulation with rhBMP7, intracellular signal transduction leads to firefly luciferase expression. The reporter system reacts to both rhBMP7 (B) and rhBMP2 (C) stimulation and the firefly luciferase signal output is proportional to the rhBMP concentration. For both rhBMP7 and rhBMP2 a concentration of 0.5 nM was selected for further experiments. (D) When co-incubating with a BMP7-antibody the luciferase signal is reduced according to the antibody concentration. A concentration of 0.5 µg/ml was selected as positive control in further experiments. (E) When co-incubating with a commercially available BMP2-antibody reduction of the luciferase signal was only observed at a high concentration of 50 µg/ml. This concentration was used as positive control for further experiments.

Following the established protocol, the BMP7 and BMP2 reporter assays were applied for IgG preparation from the fracture patients (Figure 3-15). The results were normalized to SEAP as control for cell number and metabolic activity and to the un-stimulated control (PBS). In the BMP7 reporter assay (A), stimulation with rhBMP7 shows a distinct luciferase signal (second bar). By co-incubation with the anti-BMP7-ab as positive control, the signal is completely blocked to a level similar to the un-stimulated control. Co-incubation with IgG 4, 8, 9, 10 from aAB-negative samples has no effect on the BMP7 signal transduction. Co-incubation with IgG 13, 11, 14 and 12 from BMP7-aAB positive patients blocks the signal completely to levels comparable to the anti-BMP7ab and the un-stimulated control. Co-incubation with the double positive and the BMP2-aAB positive IgG preparation reduces the signal at least for IgG 20 and 21 but do not inhibit it completely. After testing for normal distribution, t-test was performed comparing the conditions against the mean of all negatives. Significant reduction in luciferase signal was observed for all but IgG 19.

In the BMP2 reporter assay (B) stimulation with rhBMP2 shows a distinct luciferase signal as compared to the un-stimulated control. This signal is blocked by co-incubation with an anti-BMP2ab. By co-incubation with all IgG preparation no effect on the BMP2 signal propagation is observed except for the BMP7-aAB positive IgG 11. These experiments were repeated twice with different sample positions on the plate leading to the same results. However, at the moment it is not clear why this IgG preparation has a blocking effect although it proved as BMP2-aAB negative in the aAB detection assay, both with serum and IgGs.

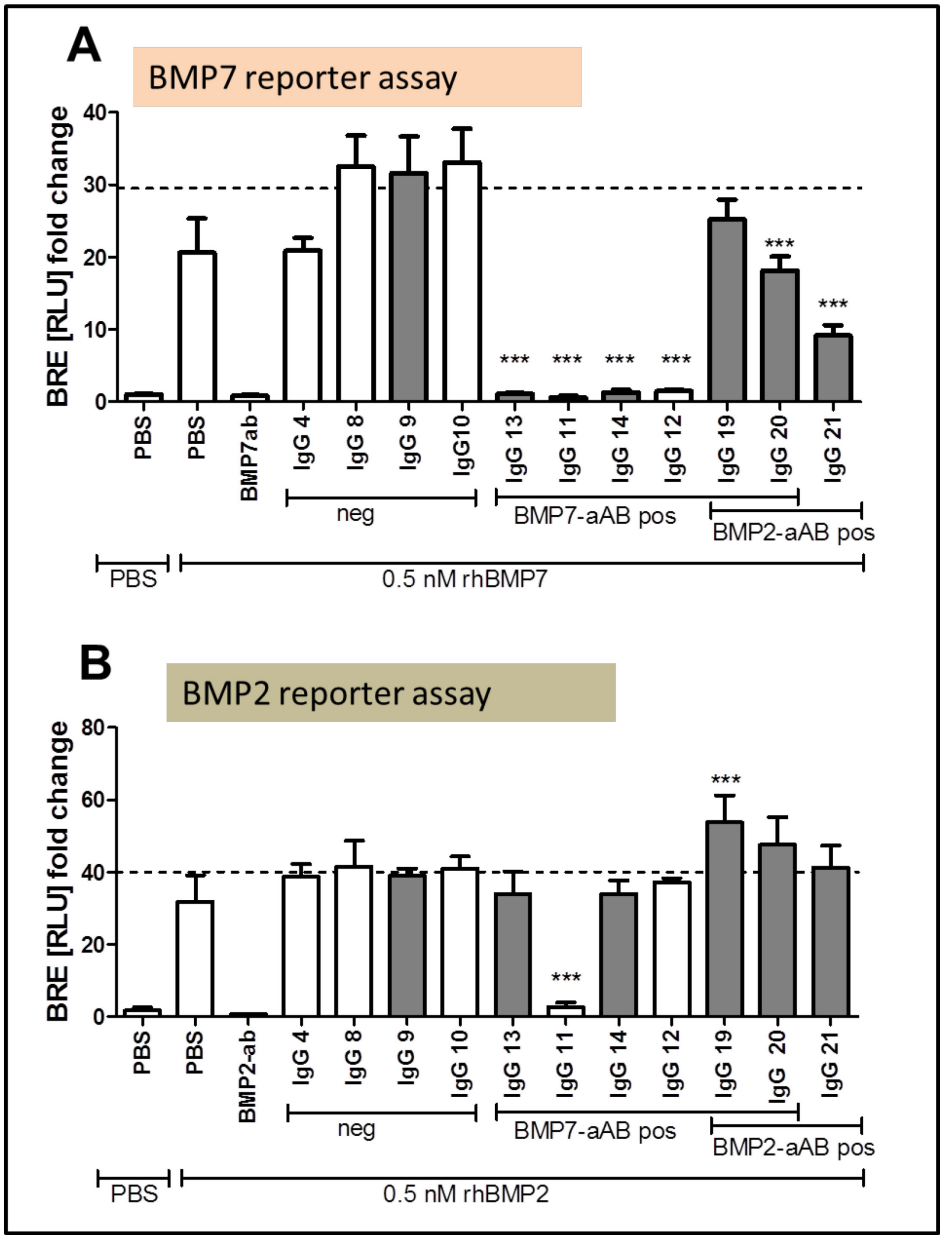

Figure 3-15: BMP reporter assay with isolated IgG preparations. NIH3T3 cells were transfected with the BRE plasmid and a secreted alkaline phosphatase plasmid for normalization (pSEAP). (A) In the BMP7 reporter assay stimulation with 0.5 nM rhBMP7 leads to a distinct luciferase signal compared to the un-stimulated control (first two bars). Co-incubation with a BMP7ab (third bar) completely blocks signal transduction. Co-incubation with IgGs from BMP7-aAB negative samples (IgG 4, IgG 8, IgG 9 and IgG 10) do not affect the luciferase signals. IgGs from BMP7-aAB positive samples (IgG 11-14) completely block the signal. IgGs from double positive samples (IgG 19 and IgG 20) show no or only little reduction of luciferase activity. Co-incubation with IgG from BMP2-aAB positive sample (IgG 21) shows a reduction in the luciferase signal but not a complete blocking of the signal. (B) In the BMP2 reporter assay a specific signal upon stimulation with 0.5 nM rhBMP2 can be detected that is completely blocked by co-incubation with a BMP2ab as positive control (first three bars). Co-stimulation with IgGs from aAB negative, BMP7-aAB positive or BMP2-aAB positive samples shows no reduction of the luciferase signal except for a single BMP7-aAB positive sample. Grey bars indicate patients that received rhBMP7 treatment. Luciferase values were normalized to the SEAP control plasmid and to the PBS-stimulated control. n = 6 (BMP7); n = 3 (BMP2); mean + SD; Student's t-test; ***, $p < 0.001$.

The results of this chapter are summarized in a schematic way in Figure 3-16. The BMP7-aAB elicit a neutralizing effect on rhBMP7. Presumably the aAB bind the rhBMP7 and prevent or disturb its interaction with the BMP receptor or the assembly of the heterodimeric BMP receptor complex. The effect in any case is that target genes are not transcribed. This effect is dependent on the strength of the aAB titres determined by the aAB detection assay and the binding characteristics of the aAB. An activating effect was not observed in any of the analysed IgG preparations.

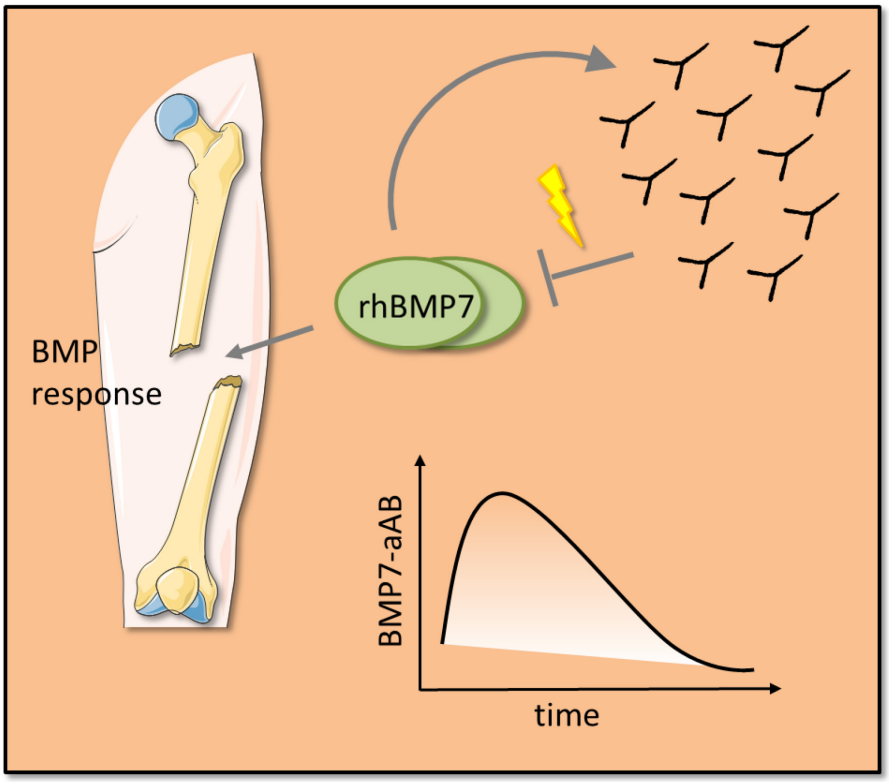

Figure 3-16: Summary of the findings by BMP-aAB measurement in fracture patients. RhBMP7-treatment of fractures induces aAB against BMP7. They have a blocking effect on BMP7 activity but diminish over time (Schuette et al., 2016).

3.5 Development and application of IGF1- and insulin-autoantibody assays

In the first chapter we realized that aAB against the IGF1R and IR are present in sera of fracture patients with the same prevalence as in other cohorts. A direct link to the fracture healing potential was not found. Therefore, the next research approach was to test whether aAB against the ligands IGF1 and insulin exist and if they have an impact on bone regeneration. Insulin-aAB are known to be present in diabetic patients (Palmer et al., 1983). The presence of IGF1-aAB has not been described before.

In order to measure aAB against IGF1 and insulin, new aAB detection assays were developed. Four different constructs were cloned, pIRES-IGF1-LUC, pIRES-IGF1-SEAP, pIRES-proinsulin-LUC and pIRES-proinsulin-SEAP, whereby the reporter gene was always positioned behind the hormone. The processing sites of proinsulin were mutated so that cleavage of the C-peptide could not take place (Figure 3-17). This enables the measurement of aAB against unprocessed insulin, which was identified as an early indicator in the progression to type 1 diabetes in children with high risk (Achenbach et al.; Yu et al., 2013).

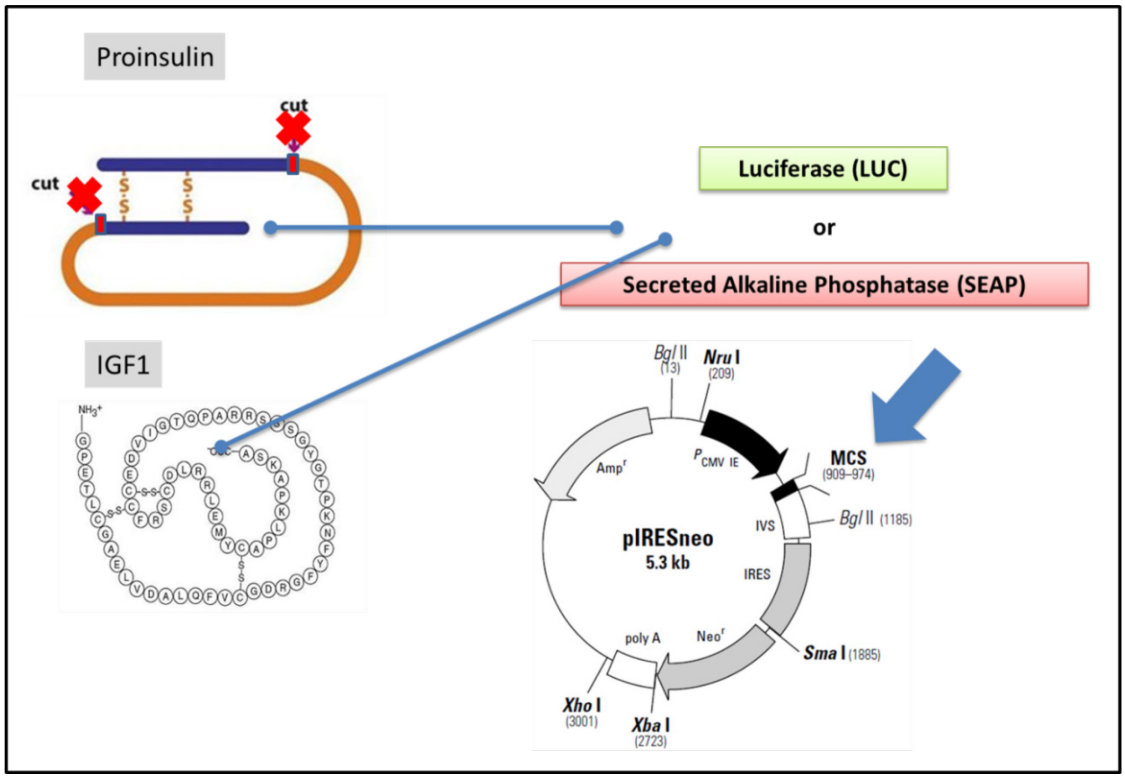

Figure 3-17: Construction of proinsulin and IGF1 reporter proteins.

HEK293 cells were transfected with these constructs and recombinant reporter proteins were expressed. Supernatants were collected and cell extracts were prepared from transfected cells. These were functionally analysed (Figure 3-18). In order to analyse whether the reporter proteins were successfully expressed, Western Blots of the extracts and supernatants were performed (A-D). Extracts from IGF1-luciferase transfected cells were analysed in an anti-LUC Western Blot (A). A distinct band at 75 kDa was detected which was not detected in the extract from untransfected cells. A corresponding section of the ponceau-stained membrane shows comparable protein loading. IGF1-LUC extract and IGF1-SEAP supernatant were further analysed via Western Blot using an anti-IGF1 primary antibody (B). Extract and supernatant from untransfected cells served as negative controls. For IGF1-LUC extract a band at 75 kDa was detected whereas the negative control did not show a signal. The IGF1-SEAP protein was detected close to BSA which is added during preparation of supernatants and shows nonspecific binding to the antibodies.

Proinsulin-LUC in extract from insulin-LUC transfected cells was detected in anti-LUC Western Blot (C). Compared to extract from untransfected cells a distinct band at 75 kDa is seen. Detection by anti-insulin antibody, Western Blot shows a clear band for insulin-LUC extract at 75 kDa but not for insulin-SEAP supernatant and not in the control extracts of both supernatant from transfected and untransfected cells (D). In summary, the successful protein expression of IGF1-LUC, IGF1-SEAP and insulin-LUC could be proven in Western Blot analysis. For IGF1-LUC and insulin-LUC, the presence was independently confirmed by anti-LUC Western Blot. The presence of insulin-SEAP could not be proven.

The recombinant proteins were further characterised by measurement of the luciferase and SEAP activity (Figure 3-18 E-H). In order to determine whether most of the recombinant protein was secreted or remained inside the cell, luciferase activity was measured in the extracts and supernatants of IGF1-LUC transfected and control cells. A high luciferase activity of 2*10^6 RLU was observed in IGF1-LUC extract compared to 1,000 RLU in control extract. Luciferase activity was low (100 RLU) for both supernatants from IGF1-LUC transfected and control cells. Hence, IGF1-luciferase proteins were not secreted but remained in the cells and the luciferase tag is functionally active (E).

Luciferase activity measurement in insulin-LUC extracts shows high luciferase activity of 1.3*10^6 RLU compared to background level of 100 RLU in control extract. In both supernatants of insulin-LUC transfected and control cells luciferase activity was at background level. The results show that insulin-LUC proteins remained in the cells and the luciferase tag is functionally active (F).

Higher SEAP activity was detected in IGF1-SEAP supernatant than in the control supernatant although SEAP activity was also detected here because cell culture supernatant on its own has phosphatase activity. SEAP activity was also higher in IGF1-SEAP extract than in control extract indicating that most of the recombinant IGF1-SEAP proteins were secreted, but a portion remained in the cell (G).

SEAP activity measurement in insulin-SEAP supernatant showed no difference to control supernatant. In both insulin-SEAP and control extracts, there was no difference in SEAP activity either (H). In addition to the Western Blot results, these results lead to the assumption that insulin-SEAP reporter proteins were not successfully expressed. Insulin-SEAP was excluded from further assay development.

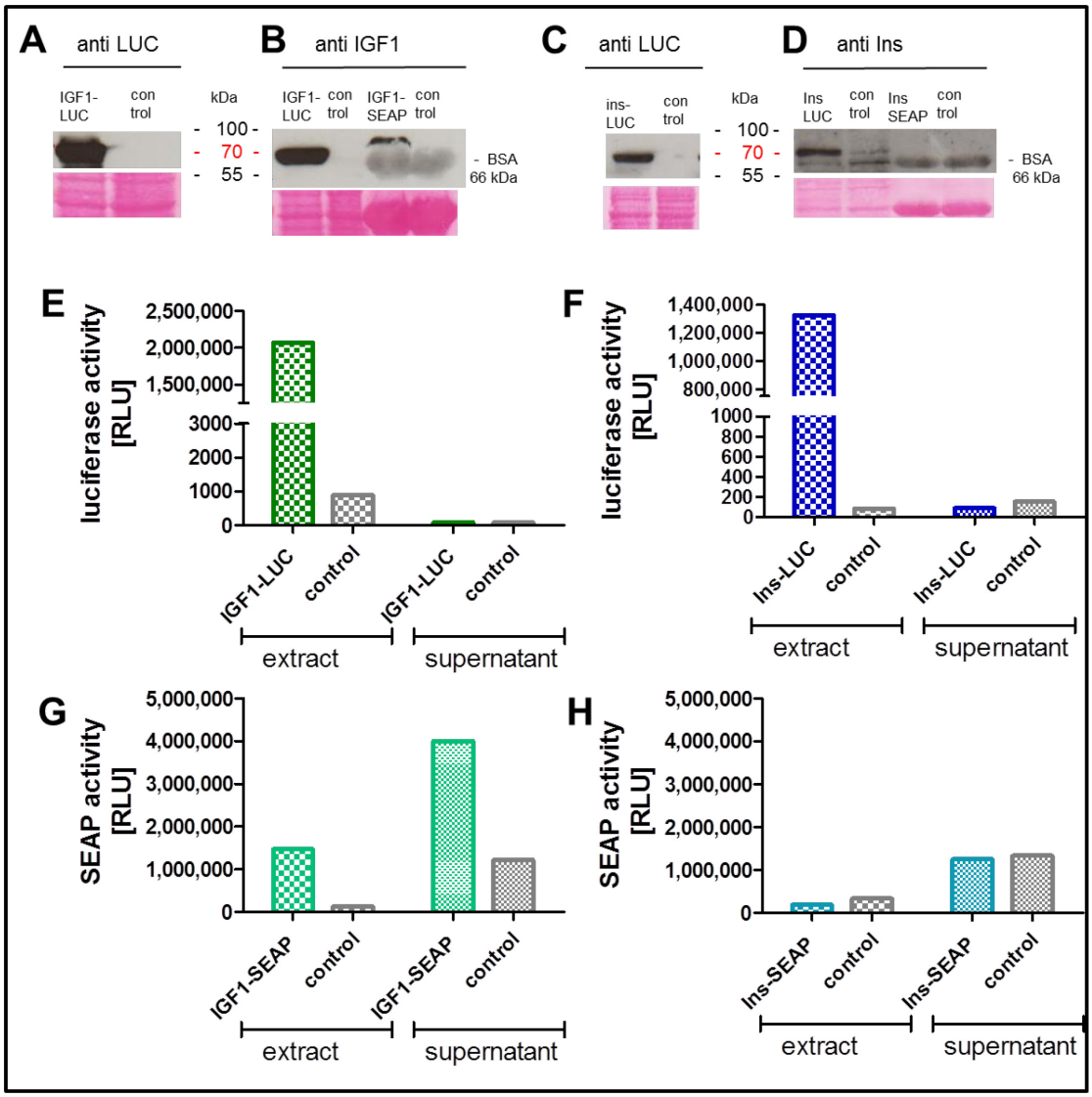

Figure 3-18: Functional analysis of IGF1-LUC, IGF1-SEAP, insulin-LUC and insulin-SEAP recombinant proteins. Analysis of IGF1-LUC extract and IGF1-SEAP supernatant showed a distinct band at approximately 75 kDa in both anti-LUC (A) and anti-IGF1 (B) Western Blots. In contrast no signal was detected in extracts and supernatants from untransfected HEK293 cells. The same section of the membrane stained with Ponceau S shows equal amounts of protein loaded to the lanes. Western blots of insulin-LUC extract detected insulin indirectly in an anti-LUC Western blot (C) and directly in an anti-insulin Western Blot (D). Insulin-SEAP on the same Western Blot was not detectable. (E) Luciferase activity measurement of IGF1-LUC extracts and supernatant shows distinct high activity in the extract. IGF1-SEAP extracts and supernatants served as controls. (F) Luciferase activity in insulin-LUC extract and supernatant also proved strong activity only in the extract indicating that the recombinant fusion proteins were not secreted but retained in the cells. (G) When measuring SEAP activity in IGF1-SEAP supernatant and extract, IGF1-LUC supernatant and extract served as controls. Strong SEAP activity was found in the supernatant but was also higher in IGF1-SEAP extract compared to IGF1-LUC extract indicating that the majority of the recombinant proteins were secreted but a portion remained in the cells. (H) SEAP activity measurement in insulin-SEAP supernatant and extract did not show higher RLU values than supernatant and extract from insulin-LUC. Together with the results of the anti-insulin Western Blot these results indicate that insulin-SEAP proteins were not properly synthesised.

To further develop the assay, the reporter proteins IGF1-LUC, IGF1-SEAP and insulin-LUC were used. Additionally, IGF1 and insulin protein was purchased, labelled with MACN and used as comparison for the self-constructed reporter proteins. In a first test, the aAB assays were performed with commercially available anti-IGF1 and anti-insulin antibodies (Figure 3-19). For IGF1-LUC (A), IGF1-MACN (B) and IGF1-SEAP (C) autoantibody assays, the anti-IGF1-antibody was detected demonstrated by high RLU values compared to negative serum and buffer control. Insulin-LUC (D) and insulin-MACN (E) aAB assays were able to detect the anti-insulin-ab with high RLU values compared to negative serum and buffer.

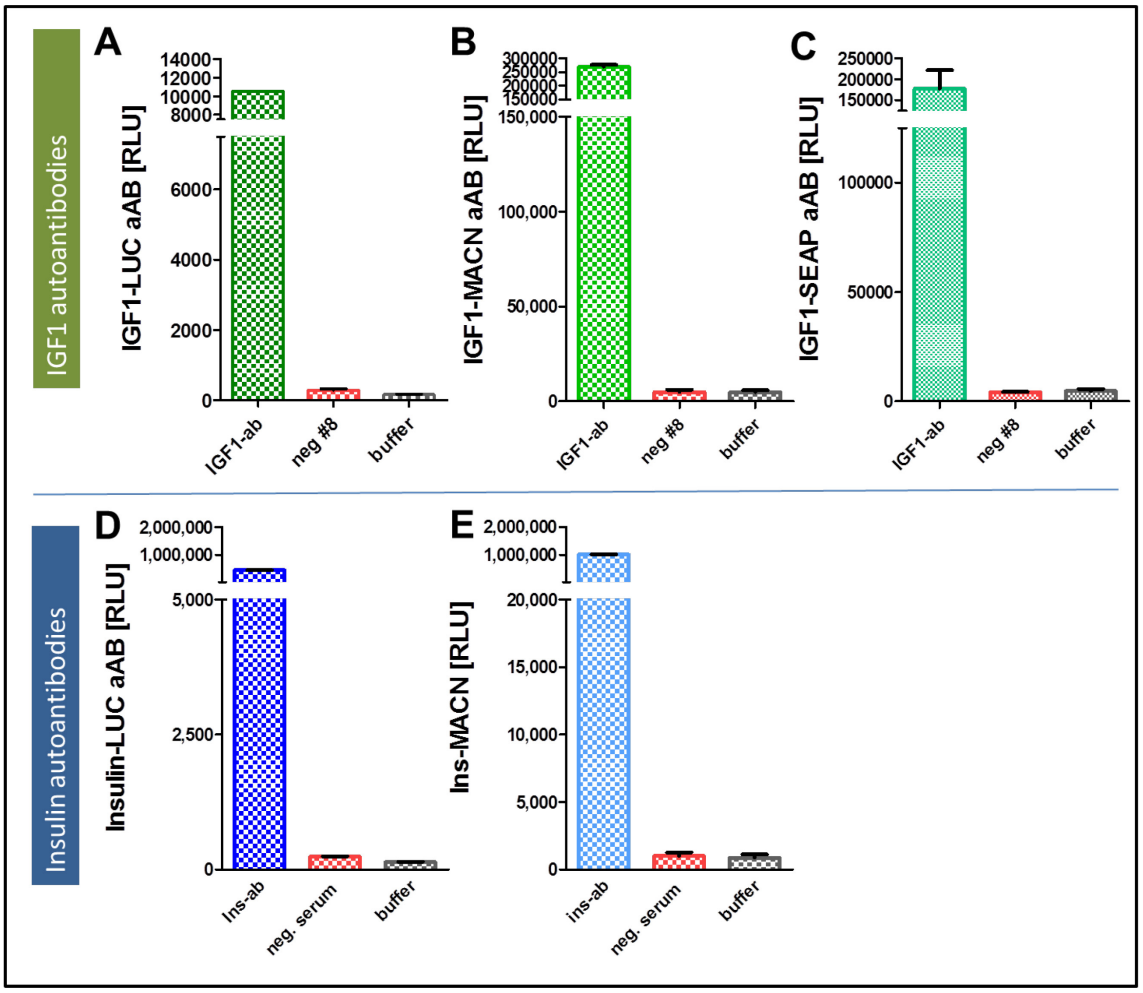

Figure 3-19: IGF1- and insulin-aAB assay test. In the IGF1-LUC (A), IGF1-MACN (B) and IGF1-SEAP (C) aAB assay a commercially available IGF1-ab can be detected (green) demonstrated by a higher signal than a negative serum (red) or buffer (grey.) When using the insulin-LUC (D) and insulin-MACN (E) aAB assays an anti-insulin-ab results in high RLU values. n = 2, mean + SD.

Since aAB against insulin are an already known phenomenon in diabetes patients, the detection assay was tested in diabetic patients. Serum samples from different diabetic phenotypes and states of insulin resistance were available (Malek et al., 2010). Insulin-aAB were measured in these serum samples by insulin-LUC and insulin-MACN aAB assay (Figure 3-20). Four samples (#202, #309, #312 and #302) clearly exceeded the cut point ($P_{0.75}$+1.5*IQR, red line) in the insulin-LUC aAB assay (A). Sample #462 was slightly elevated. The rest of the samples and the negative controls showed few variances in signal. The PBS control was lower than the negative controls pointing towards low nonspecific signal. By using the insulin-MACN aAB assay serum samples number #202, #309, #312 and #302 again were measured as insulin-aAB positive (B). The measurement of the positive samples was repeated once in both assays, with the same results.

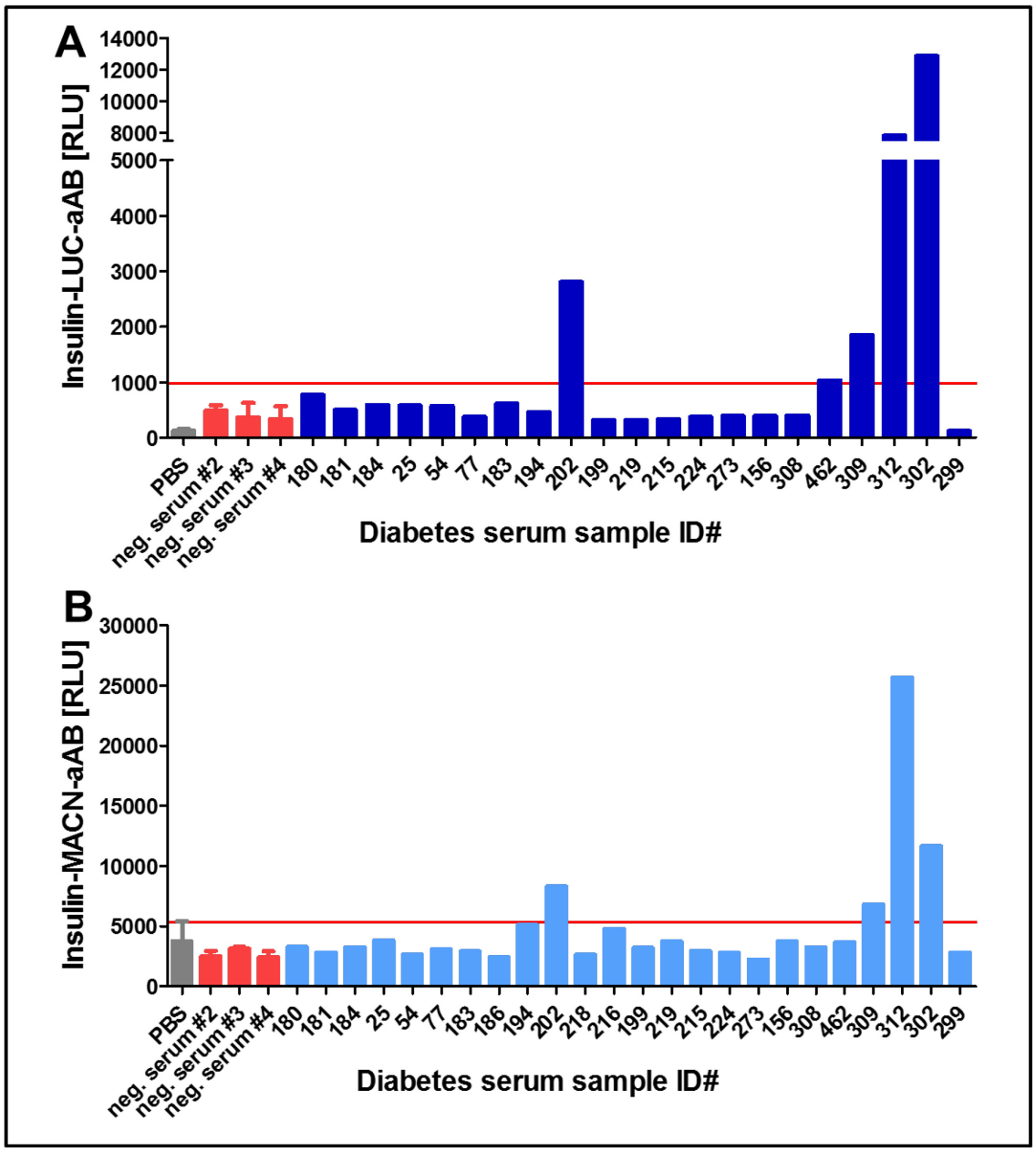

Figure 3-20: Insulin-aAB assay test in sera of diabetes patients. (A) Using the insulin-LUC aAB assay in 21 serum samples from diabetes patients, serum number #202, #462, #309, #312 and #302 showed increased values up to 14 times higher than negative controls. The other patients showed RLU signals comparable to negative control sera. (B) Measuring the same samples in the insulin-MACN assay the same result was observed at least for the high positive samples (#202, #309, #312 and #302). The cut point was set at $P_{0.75}$+1.5*IQR (red line).

In the same serum samples, aAB against IGF1 were measured with the hypothesis that IGF1-aAB could also exist in diabetes patients since insulin and IGF1 are related proteins (Figure 3-21). Using IGF1-LUC aAB assay (A) two samples (#180 and #156) showed high RLU values exceeding the cut point by 14 times. Serum sample #183 was elevated. Applying the same samples to the IGF1-MACN aAB assay (B) IGF1-aAB could not be detected in any of the samples. In the IGF1-SEAP aAB assay (C) serum samples #180, #183 and

#156 again showed elevated RLU signals which were detected as IGF1-aAB positive by IGF1-LUC aAB measurement. Additionally, #184 and #199 showed high values which were not detected as positive by IGF1-LUC measurement.
For both insulin and IGF1-aAB measurement, the LUC assays proved to have the best signal to noise ratio rather and produced reliable results. Accordingly, these were chosen for further analysis.

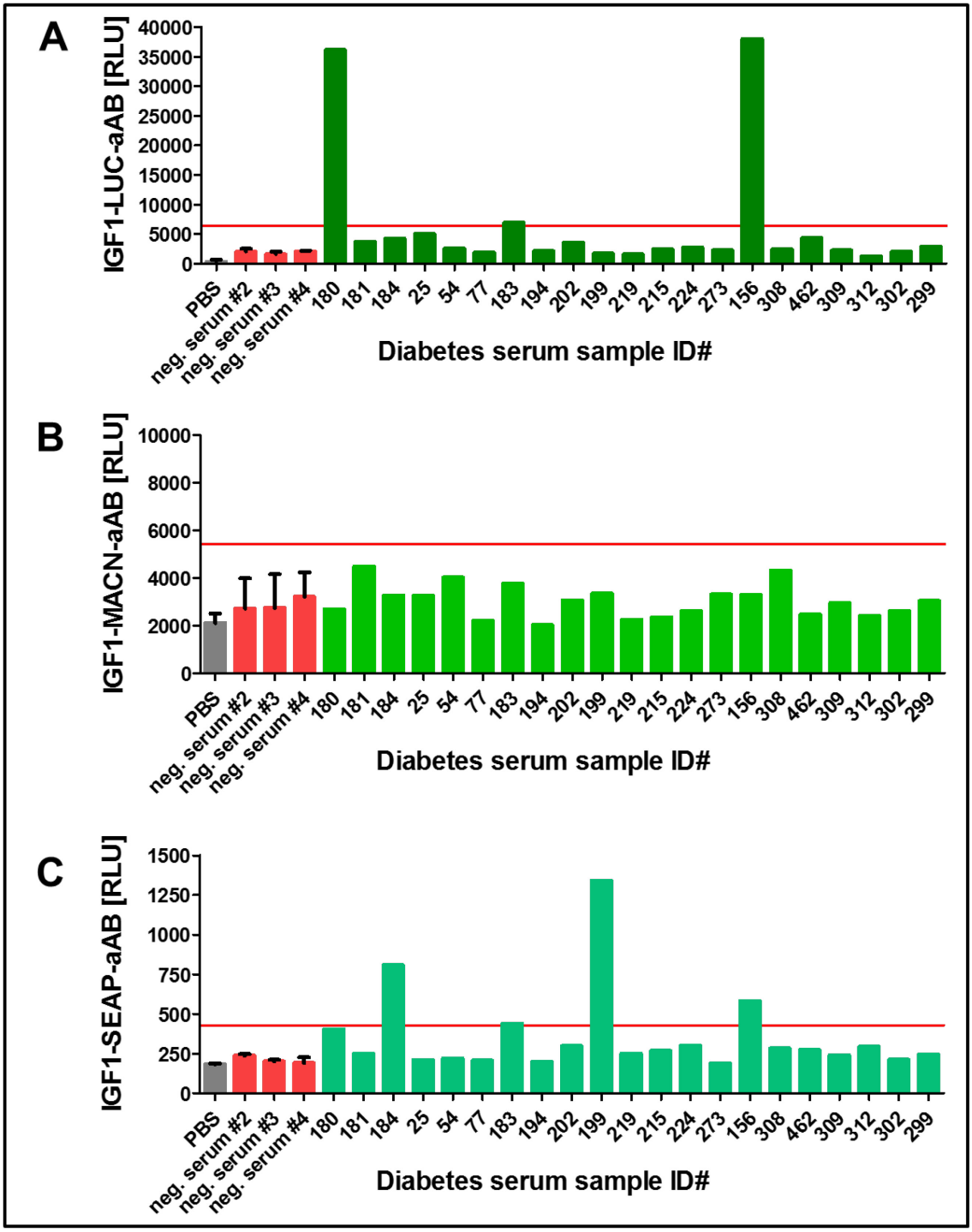

Figure 3-21: IGF1-aAB measurement in sera of diabetes patients. (A) IGF1-LUC aAB assay measurement in diabetes patients revealed a high signal in two patients (#180 and #156) and in one patient (#183) elevated values were observed. (B) Applying the IGF1-MACN aAB assay to

the same samples IGF1-aAB positivity was observed in none of the samples. (C) IGF1-SEAP aAB measurement showed aAB positivity in two patients that were not positive in the IGF1-LUC assay (#184 and #199). Patients that were positive in IGF1-LUC aAB assay (#180, #183 and #156) showed only slightly elevated signals in IGF1-SEAP aAB assay. The cut point was set at $P_{0.75}$+1.5*IQR (red line).

After thorough testing, the IGF1-LUC and insulin-LUC autoantibody assays were applied to measure IGF1- and insulin-aAB in sera of fracture patients (Figure 3-22). Measuring IGF1-aAB in 530 serum samples, 20 samples (4%) exceeded the cut point of $P_{0.75}$+1.5*IQR and were considered IGF1-aAB positive. The variance of the negative samples is rather high and the positive samples are only slightly above the cut point (A). Using the insulin-LUC aAB assay, 29 serum samples of the fracture patients were found as being insulin-aAB positive which is 6% of all samples. Titres are up to five times above the median (B). None of the positive samples was double positive for both IGF1- and insulin-aAB (C).

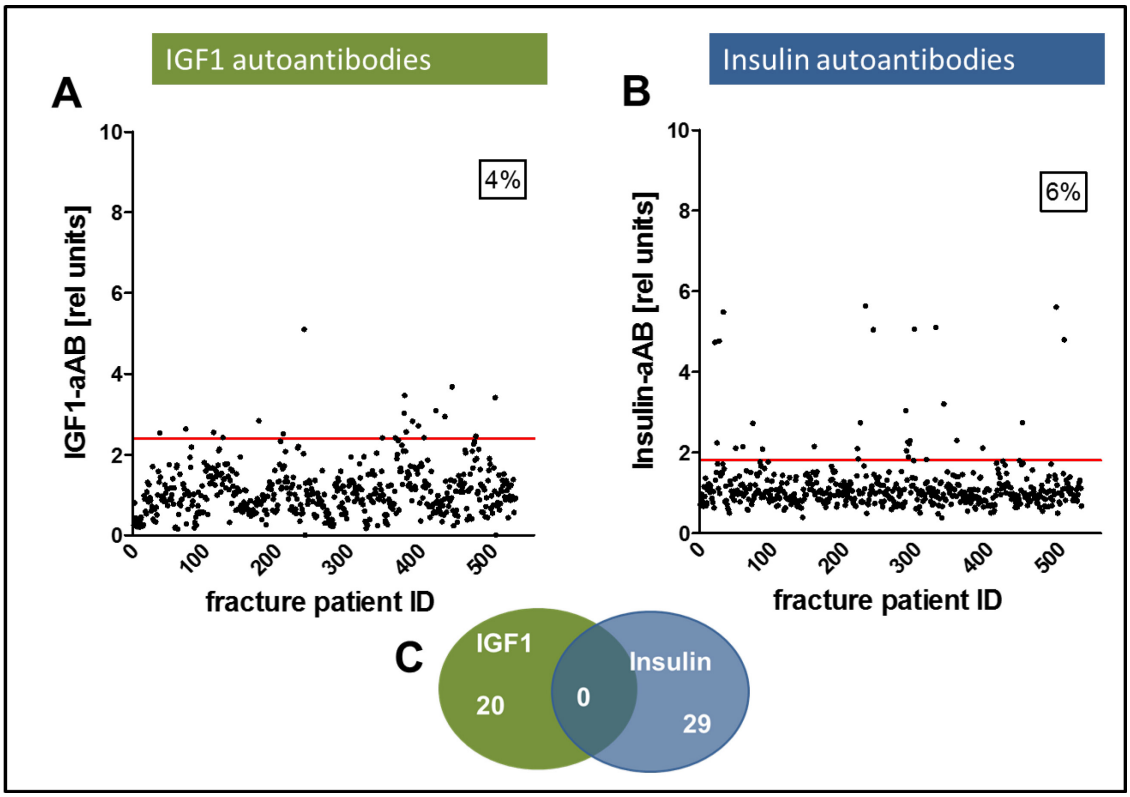

Figure 3-22: IGF1- and insulin-aAB in fracture patients. (A) Using the IGF1-LUC aAB assay 530 sera from fracture patients were measured. 20 samples (4%) exceeded the cut point (red line) and were considered IGF1-aAB positive. (B) Using the insulin-LUC assay aAB measurement revealed 29 samples (6%) as positive for insulin-aAB. (C) None of them were double positive for aAB against both proteins.

The IGF1- and insulin-aAB were further analysed at different time points after surgery (Figure 3-23). At surgery 3% of the patients were positive for IGF1-aAB and 5% of the patients four weeks after surgery (A). In insulin-aAB measurement 6% of the patients were positive at surgery and 5% four weeks later. The dotted lines show that in most cases the same patients either stayed positive or negative between the time points. Also, the titres, especially the higher ones, stayed stable in most of the patients. The results of the IGF1- and the insulin-aAB measurements are summarised in Table 3-10. Chi square analysis showed no difference of aAB occurrence between the two time points indicating that the surgery had no impact on aAB development.

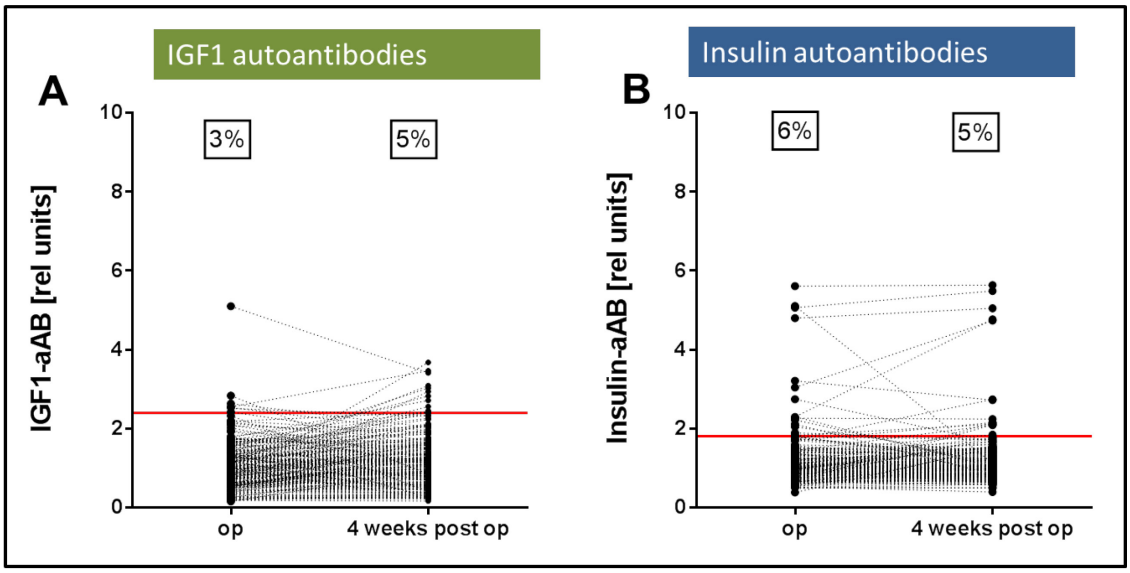

Figure 3-23: IGF1- and insulin-aAB measurement in fracture patients according to time point. (A) In the IGF1-LUC aAB assay only few samples exceeded the cut point slightly with a prevalence of 3% positive patients at surgery and 5% four weeks later. (B) In the insulin-LUC aAB assay 6% of the patients were positive for insulin-aAB at surgery and 5% four weeks later. In most cases the aAB appear rather constant with the same patient staying positive over time indicated by the dotted lines.

Table 3-10: Chi square analysis of the aAB occurrence between two time points of blood withdrawal.

	IGF1-aAB		insulin-aAB	
	op	**4 week**	**op**	**4 week**
neg [n]	258	252	250	251
pos [n]	7	13	15	14
total [n]	265	265	265	265
Pearson Chi square	$p = 0.171$		$p = 0.849$	

In a last step, it was investigated whether there is a connection between IGF1- and insulin-aAB and the healing outcome of the patients (Figure 3-24). In the consolidated group 4% of the patients are IGF1-aAB positive and in the non-consolidated group 2% are positive (A). For insulin-aAB 6% are positive in the consolidated and 4% in the non-consolidated group.

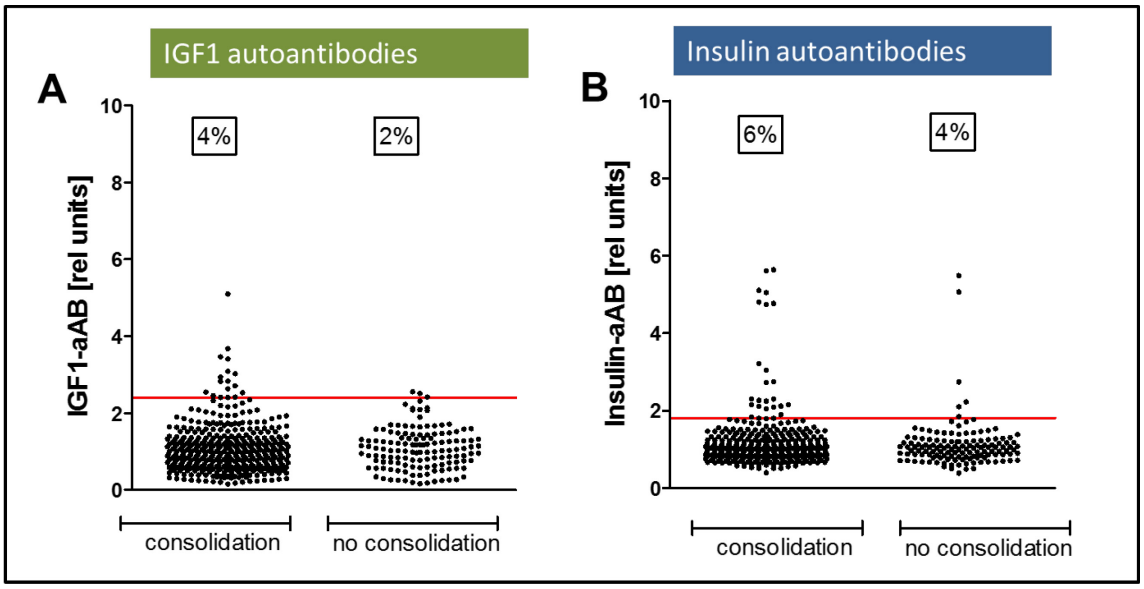

Figure 3-24: IGF1- and insulin-aAB measurement in fracture patients according to their healing outcome. (A) Using the IGF1-aAB assay 4% of the patients were IGF1-aAB positive in the group that had a positive fracture healing compared to 2% in the unhealed group. (B) Using the insulin-aAB assay 6% had insulin-aAB in the consolidated group compared to 4% in the unconsolidated group.

An overview of all the aAB positivities analysed in this study is presented in Figure 3-25. Approximately 20% of the fracture patient samples posess aAB. Most of them (16%) were detected positive for only one antigen and 5% of all samples had aAB against multiple antigens (A). A closer look on the aAB

positive samples (B) reveals different combinations of antigens (green and blue). Predominat are the aAB combinations IGF1R_IR, IGF1R_IGF1 and Ins_IGF1R_IR.

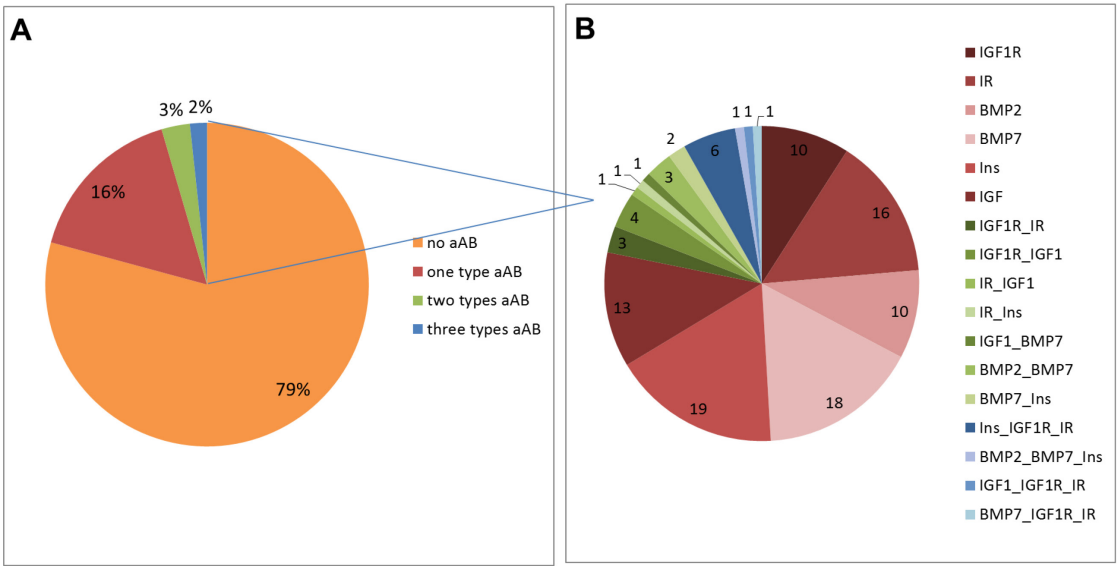

Figure 3-25: Autoantibody spectrum in sera of fracture patients. (A) In each serum sample it was analysed whether aAB against one or more of the following antigens are present; IGF1R, IR, BMP2, BMP7, Insulin and IGF1. Percentages are indicated. (B) The aAB-positive samples were further divided indicating their specific combinations of aAB positivity. Sample numbers are given.

4 Discussion

4.1 Development of autoantibody detection assays

In clinical research, it is indispensable to develop, study and characterize suitable detection assays when pursuing new research hypotheses. Autoantibody diagnostics is mostly used to diagnose and monitor autoimmune diseases or to detect and study aAB that are disease-causing. Hardly any laboratory testing is currently done on aAB that are not (yet) directly linked to a disease. Therefore, aAB detection assays for aAB that are not responsible for a certain disease are typically not developed and available on the market.

In this work, a straightforward approach for novel and potentially healing-relevant aAB was used based on the incubation of human serum with a solution of labelled antigen followed by precipitation of the immunoglobulins and detection of the emitted light units that correspond to the amount of bound aAB. Detection assays for IGF1R- and IR-aAB were already developed and validated before in our group, while detection assays for BMP7-, BMP2-, IGF1- and insulin-aAB were developed during this study. Burbelo et al. used a technically similar system of renilla-luciferase-antigen fusion proteins, which are incubated with human serum and precipitated by protein A/G beads. This proved to be a suitable approach in the detection of aAB in Sjögren´s syndrome patients (Burbelo et al., 2009).

The first precipitation assay was described in 1956 by Singer and Plotz as the latex agglutination assay, one of the first aAB detection assays at all (Singer and Plotz, 1956). They used latex particles, an antibody fraction and rheumatoid serum as a diagnostic test for rheumatoid arthritis and with that replaced sheep erythrocytes that were used before for precipitation.

A new era began when Yalow and Berson developed the radioimmunoassay. To study the insulin metabolism, they intravenously injected diabetic patients and controls with ^{131}-I-labeled insulin. What they found was that in patients who had received prior insulin treatment, ^{131}-I-labeled insulin was cleared more slowly from the circulation than in untreated patients and they showed that this was due to anti-insulin-antibodies. With their radioimmunoassay they both provided a platform to measure plasma insulin with high sensitivity and antibodies against small peptides. Yalow was given the Nobel Prize in 1977 for “the development of radioimmunoassays of peptide hormones”. In

endocrinology, this technology is still widely applied due to its high sensitivity in the detection of hormones and measurement of their activities.

Later, enzyme-linked immunosorbent assays (ELISA) were developed for the detection of aAB, as well as other techniques such as chemiluminescence immunoassays (CIA) and surface plasmon resonance (SPR). Today companies offer a wide range of different assay systems as diagnostic tools for autoimmune diseases and for disease-causing aAB.

Here, a relative simple and robust approach was used based on immuno-precipitation. Assay control and validation steps were performed as it is recommended for development of immunoassays (Mire-Sluis et al., 2004; Shankar et al., 2008). The output of the assays is relative light units of the antigen-label which was converted in a positive/negative format by defining a floating cut point. Antibodies derived from animals immunized with the human antigens served as positive controls. Dilution series of antibodies and positive or negative sera showed linearity of signal intensity. Quality controls were used, i.e. an antibody as positive control, control sera and PBS in assay buffer. These controls were applied at the beginning and end of each run.

In order to characterize the BMP-aAB, their effect on BMP signal transduction was analysed via a cell-based assay which also provided an independent verification method for the precipitation assay. Requirements for a cell-based assay are (a) a drug-responsive cell-line, (b) the drug, in this case the biological BMP, (c) a positive control antibody and (d) test species serum (Gupta et al., 2007; Gupta et al., 2011). The assay endpoint is transcription of target genes which is considered a late endpoint together with other possible outputs such as cell proliferation or cell differentiation, as opposed to early endpoints such as binding of drug to the receptor or receptor phosphorylation. The BMP reporter assay can test for agonistic and antagonistic properties of the aAB. For both the control antibodies and the sera tested, antagonistic activity was observed. The reporter system is not specific for BMP7 but also reacts upon BMP2 stimulation. This promiscuity was used to determine the specificity of the aAB. BMP7-aAB were only able to suppress the reporter activity when the system was stimulated with BMP7 and not when it was stimulated with BMP2.

4.2 Autoantibodies against the insulin and IGF1-axis

One other study from our group, using exactly the same detection assay but slightly different cut off criterion for positivity, analysed IGF1R-aAB in Graves´ Orbitopathy patients and healthy controls (Minich et al., 2012). By applying the mean + 3SD they found about 10% IGF1R-aAB positive patients and controls which is in line with the findings in fracture patients in this study. According to this result the cohort of fracture patients does not *per se* show a higher prevalence of IGF1R autoimmunity. Similarly, they reported steady levels of aAB titres over time for three time points per patients that were approximately six months apart.

In analysing a cohort of over 200 control sera for IR- and IGF1R-aAB and their cross-reactivity, 5% IR-aAB and 6% IGF1R-aAB positive individuals were identified (Welsink Tim, 2013). Again, our findings of IR-aAB prevalence in fracture patients (5%) are comparable to this result in healthy controls. Cross-reactivity between aAB against both receptors was seen in 50% of positive samples as it was observed in the fracture patients.

IgG preparations from IGF1R-aAB positive samples were biologically active demonstrated by blocking of IGF1-induced receptor phosphorylation (Minich et al., 2012). Also, an effect on cell growth was reported. The number of viable cells was reduced when breast cancer cells were co-incubated with IGF1 and IgG from aAB positive samples compared to co-incubation with IgG from aAB negative samples. This leads to the assumption that also the IGF1R-aAB detected in fracture patients are biologically active probably in an antagonistic fashion, thus potentially affecting IGF1 signalling. However, direct effects of IGF1R-aAB on the overall regeneration, i.e. the bone healing outcome, was not observed. A reason for this lack of interaction might be that the blocking effect becomes compensated for by expressing more receptors or secreting more IGF1. Additionally, cross-reactivity between insulin and IGF1 and their receptors can occur making the regulation even more complex.

Novel assays for the detection of proinsulin- and IGF1-aAB were developed based on the incubation of human serum with recombinant antigen-luciferase proteins, followed by precipitation of immunoglobulins and measurement of the emitted light units of the luciferase tag. The recombinant fusion proteins were successfully expressed in cell culture and the luciferase tags were active. The

aAB assays were established by the use of commercial anti-insulin and anti-IGF1 antibodies using the technology described before for the other aAB detection assays.

In this work, insulin-aAB were found in serum samples of diabetic patients as were IGF1-aAB. This is the first time that IGF1-aAB are described. In the literature, the presence of insulin-aAB was associated with diabetes and they were found to be early indicator for islet autoimmunity in the development of type 1 diabetes. Especially, proinsulin-aAB are the earliest prediabetic predictors (Yu, Dong et al. 2013). In children, the age when islet-aAB were detected, were found to predict the age of diabetes diagnosis (Steck, Johnson et al. 2011). Compared to MACN-labelled proteins, the newly developed assays using fusion proteins showed comparable results, i.e. the same samples were detected as aAB positive but the discrimination between positive and negative was clearer.

In fracture patients insulin- and IGF1-aAB were identified with a prevalence of 4% and 6%, respectively. This is so far the first time that insulin- and IGF1-aAB are addressed in bone healing. A change of prevalence between two time points of blood withdrawal, at surgery and four weeks later, was not observed and a direct effect on fracture gap consolidation was not recorded. So far, it can only be stated that these aAB exist, their impact (or whether they have an impact) on bone healing remains to be elucidated.

4.3 Natural BMP-autoantibodies

The prevalence of 2.5% for BMP7-aAB in healthy adults is in line with a previous study that described a prevalence of 1.6% and 3% of naturally occurring aAB against BMP7 in subjects younger or older than 65 years of age (Sauerborn et al., 2011). Collectively, these data indicate that a small fraction of healthy adults are already positive for BMP7-aAB despite not having been exposed to rhBMP7 before in their life. In contrast to the analysis presented here, the former study reported a prevalence of up to 50% in BMP7-treated patients (Sauerborn et al., 2011). This value is much higher than observed here, where up to 18% of patients proved positive for BMP7-aAB after rhBMP7 exposure. The reasons for this discrepancy are unclear, but might lie in the

different treatment regimen applied, the different assays used or the criteria applied for defining positivity.

Both studies highlight that natural BMP7-aAB exist and that BMP7-aAB can be induced by rhBMP7 treatment. This autoimmune response to the applied rhBMP7 does not develop in all of the treated patients, but in a subset. The parameters predisposing some patients to BMP7-aAB are probably similar to those determining the risk of autoimmunity in the general population, including genetic predisposition (Perricone et al., 2013), obesity (Versini et al., 2014), smoking status (Perricone et al., 2016) and other environmental factors. Specifically, B cells are primed to be activated upon a sudden increase in antigen along with inflammatory signals (cytokines) whereas constant antigen signals in absence of inflammation may rather be tolerated (Janeway, 1992; Matzinger, 2002).

Comparing the aAB against BMP2, the former study reported a prevalence of 2.3% in subjects below 65 years of age, and 5.3% in subjects who are older (Sauerborn et al., 2011). Again, these data are well in agreement with the results in fracture patients and controls which were 2-6% BMP2-aAB positive subjects. Notably, the prevalence of BMP2-aAB positive patients was higher in samples collected after rhBMP7 treatment, however the titres of these aAB were relatively low and close to the cut-off level. These data imply a high specificity of therapy-induced aAB to the actual biological agent used with little cross-reactivity to a related growth factor (Schuette et al., 2016).

4.4 Anti-drug antibodies

Throughout this work, all antibodies detected in patients´ sera were termed autoantibodies. In those patients that were treated with rhBMP7, the detection of BMP7 antibodies is in fact a detection of anti-drug antibodies. Therefore, the induction of anti-drug antibodies will be discussed briefly in the following.

One of the first studies detecting anti-drug antibodies was the aforementioned pivotal study by Yalow and Berson where they found antibodies against insulin in patients that had received insulin treatment (Berson et al., 1956). Contrary to the predominant opinion it was shown herein that small peptides are antigenic. Later drugs were designed less immunogenic by making them more similar to human proteins and improved biotechnological production. The application of

recombinant endogenous proteins (biologicals) as therapeutics reduces the immunogenicity to a minimum but bears the additional risk of cross-reactivity of a potential immune reaction to the endogenous counterpart.

Considering the three main concerns regarding anti-drug antibodies of biologicals, i.e., (1) neutralization of the drug, (2) immune reaction by the drug-AB complexes and (3) cross-reactivity of the ab to the endogenous protein, the first issue seems to apply to treatment-induced BMP7-aAB as they neutralized the activity of the recombinant protein in vitro and prevented it from activating its signalling cascade. However, whether the neutralizing effect has any strong and relevant clinical consequences could not be fully evaluated in this study due to the heterogeneity and limited number of aAB-positive subjects. Analysing consolidation as the most relevant end-point of the treatments, the results indicate that indeed there was a higher number of BMP7-aAB patients who failed to reach successful fracture gap closure, however, the subjects with the highest BMP7-aAB titres were found in the group of successfully treated patients.

Cross-reactivity of the BMP7-aAB to the endogenous protein is likely to occur because the drug is a recombinant human protein variant with high similarity to the endogenous form. However, the short transient nature of the induced BMP7-aAB peaks argues against adverse long term consequences, except for situations in which the antigen is again provided, e.g. upon repeated therapy with the biological in case that fracture healing failed.

Antibody formation against biologicals has long been known, e.g. during the course of anti-TNFα antibody Infliximab (Steenholdt et al., 2013), interferon-β (IFN-β) (Scagnolari et al., 2002; Sorensen et al., 2003; Aarskog et al., 2009), erythropoietin (EPO) (Casadevall et al., 2002) or thrombopoietin (THPO) (Li et al., 2001) treatment. In all of these applications, it was shown that antibodies are capable of interfering with the efficacy of the drug. EPO antibodies were associated with the severe consequence of pure red cell aplasia (Casadevall et al., 2002). Antibody development against THPO was associated with thrombocytopenia due to cross-reaction with the endogenous THPO. The incidence of thrombocytopenia was higher in healthy volunteers than in immune compromised cancer patients, and similar to the BMP7-aAB in this study, also the antibodies against THPO disappeared over time (Li et al., 2001). Anti-IFN-β

antibodies are extensively studied to better characterize their occurrence and effects, as IFN-β is used as treatment for multiple sclerosis (Sauerborn et al., 2011; Kijanka et al., 2015; Haji Abdolvahab et al., 2016). The result of anti-Infliximab antibodies is loss of treatment response. Comparable as in this study, pre-existing Infliximab-antibodies were reported (Steenholdt et al., 2013). A correlation of these with the treatment outcome was recorded.

However, the mechanisms underlying the patient-specific responses to the biologicals are still far from being understood. All of the treatments mentioned above are given systemically and over a longer period of time, in contrast to the rhBMP7, which is applied locally into the fracture gap usually only once. Here, a relatively high amount of rhBMP7 is suddenly present initiating a fast biological response but its stability and the amount that is actively involved in bone regeneration is difficult to estimate. In the same line, aAB induced by the treatment are not characterized by a stable titre and are equally difficult to be assessed for their potentially interfering capacity and clinical importance.

The application of rhBMP7 in fracture patients provides an unprecedented antigen surge into an inflamed environment. Patients that contain autoreactive B cells which are present in immunological tolerant form may become activated especially in the context of an ongoing inflammation. These aspects as well as the genetic predisposition to develop autoimmunity vary between individuals and my account for the heterogeneous picture observed (Schuette et al., 2016). Further studies along this line are needed.

4.5 Clinical relevance

Clinical relevance of aAB is given without doubt when they are the responsible agents for autoimmune disease or present in the majority of patients. Researchers have tried to draw a line between disease-causing aAB and aAB that are merely bystander. Therefore, Naparsteck et al. have defined criteria for pathogenesis, i.e. (1) aAB should show the same effects in an experimental setting, (2) immunization that leads to similar antibodies should lead to similar symptoms, (3) aAB should be found together with the antigen at the site of tissue damage, (4) aAB titre and disease severity should correlate and (5) removal of aAB e.g. by plasmapheresis should diminish the disease symptoms (Naparstek and Plotz, 1993).

To investigate aAB that are not disease causing and to define when they become harmful is an even more challenging task not least because we need to define harmful first. An easy approach would be to define a pathological titre but then again we need to know at which titre negative side effects occur. Consequently, aAB have to be considered as harmless unless any impact can be observed. Since the output of the signalling pathways of IGF1 and insulin are manifold it must be noted that although no negative consequences in patients carrying aAB were observed in this study it might be possible that the relevant endpoint was not addressed.

For anti-drug antibodies negative impacts can be defined more easily. First, a neutralizing effect of the ab would be a prerequisite and was shown during this work for anti-BMP7-ab. An effect of the treatment efficacy would be the next measurable outcome. When aAB inhibit treatment efficacy they reached a clinical relevant activity. The next consideration is regarding safety. To this end, the BMP7-aAB trend over time was measured up to one year post surgery. In nearly all cases of positive subjects, aAB titres dropped rapidly to undetectable levels obliterating concerns regarding long-time effects. However, it remains obscure whether repeated treatment in case of insufficient healing would boost the immune system similar to a booster immunization and would lead to a stronger and long-lasting response. Clinical consequences such as activation of the immune system by the aAB could not be analysed during this work but should be kept in mind. Nevertheless, it was demonstrated here that in any given sample different combinations of aAB exist. Although clinical relevancies were not found observing one antigen, it might be that the combination of different aAB specificities indeed leads to a clinical phenotype. On a logistical basis, only a limited number of antigens could be analysed by our method. Therefore, to investigate the aAB spectrum in each patient, a large panel of antigens needs to be measured and analysed for clinical association, for example via multiplex screening.

4.6 Autoimmunity – not a failure of the immune system but a normal phenomenon?

The original dogma stated that the immune system is only directed against foreign substances to protect all self-moieties for which it is tolerated. This was

challenged by the identification that the immune system can be directed against self leading to a pathological state of autoimmune disease. Recent reports support the notion that self-reactivity occurs and does not necessarily lead to pathological consequences. The present work also contributes towards this direction by showing that firstly, aAB occur in apparently healthy individuals and secondly, even without any obvious consequences.

The self/non-self recognition and clonal selection theory by Burnet predominated the autoimmune field for a very long time and overruled all other concept regarding the regulation and activation of the immune system (Burnet, 1959). Unable to explain all occurring phenomena the self/non-self dogma was shaped or extended to infectious versus non-infectious (Janeway, 1992) and even to harmless versus dangerous antigens (Matzinger, 2002). These theories however still discriminate between categories. It might be necessary to revise our view of immune activation which is dominated by the idea of an army ready to attack harmful invaders. The immune system might be part of the normal homeostasis of the organisms and actively contributing in maintaining it. A set of natural aAB mainly of the IgM class are present from birth and are also found in mice that were held in antigen-free conditions (Coutinho et al., 1995). A regulatory function of the immune system is, e.g. provided by these regulatory natural aAB in the clearance of apoptotic cells. Patients receiving immunosuppressive treatment have a higher chance of de novo tumour development indicating a tumour-protective role of the immune system (Penn and Starzl, 1973). Taken together, the immune system might rather be seen as regulatory entity than a collection of defence mechanisms. Other global concepts were described such as incorporation as opposed to tolerance (Parnes, 2004). Pradeu et al. defined continuity as a criterion for immunogenicity (Pradeu and Carosella, 2006). The theory of immunological homunculus in analogy to the neurological homunculus states that self-reactivity is required for proper defence of microbial disease and attributes the immune system with the ability of monitoring and controlling the antigen state of the body in healthy and diseased condition. To this end, it requires an “immunologic reflection-image of the body state”. Similar to the endocrine and neural system the concept postulates homeostasis by feed-back-loops and “images”. The function of the immune system might be to “tidy up” dangerous accumulations

of self-antigen produced by tissue damage, apoptosis, senescence or foreign invasion. In any case, theories struggle in explaining why an unborn foetus is tolerated but a transplanted organ is rejected, a microbial infection is defended but food intake and commensal bacteria are tolerated, after all why some individuals develop autoimmune disease and why some carry aAB at low titres without negative consequences although constant availability of the self-antigen.

4.7 Conclusion

Autoantibodies were identified in healthy controls and fracture patients. BMP7-aAB have been detected as pre-existing and rhBMP7 therapy induced antibodies and are neutralizing in nature potentially effecting osteogenic differentiation. This phenomenon may be of clinical importance as some patients fail to respond to the fracture healing treatment for as yet unknown reasons. Induced BMP7-aAB were seen to disappear over time as opposed to aAB against the insulin and IGF1-axis which appeared to show more steady titres over time. However, a direct negative impact on bone healing was not recorded by analysing consolidation of the fracture gap as the most-relevant end point. Collectively, these results do not support concerns regarding the presence of aAB against growth factors and their receptors and the safety of BMP7 treatment to improve bone healing in general, however, individual-specific effects cannot be ruled out as the spectrum of different aAB in a given subject appears as diverse and heterogeneous as the individual genome.

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Publications

Peer-reviewed publications

Andrea Schuette, Arash Mogghadam, Petra Seemann, Georg N. Duda, Gerhard Schmidmaier, Lutz Schomburg; *Treatment with recombinant human bone morphogenetic protein 7 leads to a transient induction of neutralizing autoantibodies in a subset of patients*; BBA Clin.; 2016

Sarah Blasig, Peter Kühnen, **Andrea Schuette**, Oliver Blankenstein, Jens Mittag Lutz Schomburg; *Positive Association of Thyroid Hormones and Serum Copper in Children with Congenital Hypothyroidism*; Journal of trace elements in medicine and biology; 2016

Zevenbergen, Chantal*; Korevaar, Tim*; **Schuette, Andrea***; Peeters, Robin; Medici, Marco; Visser, Theo J.; Schomburg, Lutz; Visser, W. Edward, A*ssociations of antiepileptic drug usage, trace element and thyroid hormone status*; Eur J Endocrinol; 2015 *authors contributed equally

Oral presentations

Andrea Schuette, Arash Moghaddam, Petra Seemann, Gerhard Schmidmaier, Lutz Schomburg. *Autoantibodies against bone morphogenetic protein 7 in fracture patients*. 10th International Congress on Autoimmunity. Leipzig, Germany. 6-10 April 2016.

Poster

Andrea Schuette, Arash Moghaddam, Petra Seemann, Gerhard Schmidmaier, Lutz Schomburg. *Neutralizing antibodies against bone morphogenetic protein 7 in fracture patients*. World Congress on Osteoporosis, Osteoarthritis and Musculoskeletal Diseases. Málaga, Spain, 14-17 April 2016.

Andrea Schuette, Waldemar Minich, Tim Welsink, Christian Schwiebert, Petra Seemann, Arash Moghaddam, Gerhard Schmidmaier, Lutz Schomburg. *Autoantibodies and bone regeneration*. International Symposium on Bone Regeneration. Berlin, Germany. 11-13 June 2015.

Schuette A, Minich WB, Welsink T, Schwiebert C, Brown RJ, Gorden P, Schomburg L. *Prevalence of autoantibodies to the insulin and IGF1 receptor*. 57. Symposium der Deutschen Gesellschaft für Endokrinologie. 2014. Dresden. Germany. 19-22 march 2014.

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit mit dem Titel „Autoantibodies against growth factors and their receptors in fracture healing“ selbstständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.
Des Weiteren erkläre ich meine Kenntnisnahme der dem angestrebten Abschluss zugrunde liegenden Promotionsordnung. Ich versichere, dass ich die vorliegende Arbeit weder in dieser noch in einer anderen Form bei einer anderen Prüfungsbehörde eingereicht habe und dass ich nicht im Besitz eines entsprechenden Doktorgrades bin.

Berlin,

_____

Andrea Schütte